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Modulation of immune responses by UV irradiation

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Edinburgh, UK

Lay summary

Atopic dermatitis (AD) is a chronic disease, the prevalence of which has been rising in Western society and now affects more than 1 in 5 people. Both genetic factors and abnormal immune responses are known to contribute to the onset and persistence of AD. The filaggrin gene is mutated in around 40% of AD patients. This contributes to the formation of skin barrier against environment, but it has also been associated with immunological abnormalities. AD improves markedly on exposure to UV light and this is used to treat severe AD, yet the mechanism of action remains elusive. UV prevents excess immune cells infiltrating skin and also induces cell death of the immune cells that are already present in lesional AD skin. Besides its direct effect on immune cells, UV increases vitamin D levels and vitamin D level has been shown to be low in children with severe AD. UV also converts trans-urocanic acid, which is the breakdown product of filaggrin, to *cis*-urocanic acid (*cis*-UCA). This suppresses induction of allergic responses to contact sensitizers. UV releases NO, which also affects immune cells. Vitamin D can reduce inflammation by increasing a type of immune cell called T regulatory cells (Tregs). The biological function of vitamin D is mediated by vitamin D receptor (VDR). All agents are induced by UV although the relative importance of each is not understood, nor is their interaction. Therefore, I investigated the effect of NO and *cis*-UCA on immune cells derived from peripheral blood from healthy volunteers. I also investigated the correlation between plasma concentration of 25(OH) vitamin D and nitrate, FLG genotype, circulating Tregs and clinical efficacy of NB-UVB phototherapy.

Results showed that NO directly promoted the generation of Tregs with enhanced capacity to migrate to the skin. The NO-induced Tregs modulated immune response through membrane bound TGFβ and PD-1/PD-L1 but not CTLA-4. *Cis*-UCA might promote inflammation by

decreasing Tregs and VDR expression. *Cis*-UCA might also modulate inflammation by inhibiting CD4+T cell migration, enhancing immune suppressive cytokine secretion, decreasing CD4+T cell proliferation, inhibiting cell signalling pathway and promoting the generation of Tregs indirectly. Results generated from the clinical study suggested that although circulating 25 (OH) vitamin D concentration was significantly increased after NB-UVB phototherapy, the change of circulating 25 (OH) vitamin D concentration did not correlate with disease improvement. This suggested that plasma 25 (OH) vitamin D concentration could not be used as biomarker to predict clinical efficacy of NB-UVB phototherapy. Changes of plasma nitrate concentration and FLG genotype could also not be used as biomarker for this purpose. The expression of markers reflecting T cell activation and migration were decreased after treatment. Their importance in determining clinical responses requires further investigation.

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Abstract

Atopic dermatitis (AD) is a common, chronic relapsing inflammatory skin disease associated with cutaneous hyper-reactivity to environmental triggers that are innocuous to normal non-atopic individuals. AD affects 10% to 15% of children and 2% to 10% of adults in industrialized countries. There has been increasing interest in this disease triggered by its increasing prevalence in western societies and its contribution to the increasing health care costs. Yet, the underlying pathophysiologic and genetic mechanisms leading to the manifestation of AD are not clear. AD results from a complex interplay between environmental triggers, susceptibility genes including mutations in the keratinocyte protein filaggrin and altered immune responses resulting in allergic CD4+ T cell (Th2) immunity to epidermally encountered antigens. Regulatory T cells (Tregs) play an important role in controlling responsiveness to self-antigens and preventing autoimmune diseases, as well as in limiting inflammatory responses during inflammation and infection. Currently, studies investigating the number and function of Tregs in patients with AD have shown controversial results.

It has been long established that symptoms of AD improve on exposure to sunlight. Narrowband UVB (NB-UVB) phototherapy is a common treatment modality for a variety of skin diseases. Considering the adverse effects for systemic treatment for severe adult AD, phototherapy, especially NB-UVB phototherapy may be a more practical long-term treatment. However, approximately 50% of patients over an 8-week treatment course do not improve after NB-UVB phototherapy. Therefore, it is important to identify characteristics of AD patients to determine whether they will respond to phototherapy and to avoid adverse effects for unresponsive patients.

UVB exposure has also been associated with induction of Tregs in mice and increasing their numbers and/or functional capacity may offer benefit to patients with chronic AD. Active vitamin D ($1,25(OH)_2D_3$), one of the factors induced by UV-B radiation induces Tregs and is suggested to contribute to the suppressive effect of NB-UVB phototherapy. However, UV radiation could also have beneficial effects through other pathways known to affect immunoregulation. UVB exposure upregulates production of nitric oxide (NO) in the skin which also affects immune cell function. The protein filaggrin is broken down in differentiating keratinocytes to form the natural moisturizer of the skin. The gene encoding filaggrin (FLG) has been shown to be a major predisposing factor for AD. A key breakdown product is urocanic acid (UCA) which also acts as a natural sunscreen and undergoes trans-cis isomerisation on exposure to UV-B. *Cis*-UCA is known to modulate immune responses, however, the mechanisms of its action remain elusive. The production of all three compounds, vitamin D, *cis*-UCA and NO might all increase in the circulation of patients undergoing UVB phototherapy. While the immunomodulatory effect of Vitamin D is well described, *cis*-UCA and NO may also affect the behaviour of T lymphocytes systemically. Therefore, I investigated the effect of NO and *cis*-UCA on the phenotype and function of CD4+T cells and monocyte-derived dendritic cells (Mo-DCs) derived from peripheral blood mononuclear cells (PBMCs) from healthy volunteers. I also investigated the correlation between plasma concentration of 25(OH) vitamin D and nitrate, FLG genotype, circulating Tregs and clinical efficacy of NB-UVB phototherapy.

My results showed that NO did not affect the phenotype of human mo-DCs and directly affected peripheral CD4+ T cells by inducing functional CD25+Foxp3+CD127-Tregs from CD4+CD25lo/- effector T cells. Moreover, NO increased expression of the of skin homing marker CLA on these Tregs, suggesting an increased ability of NO-induced Tregs to migrate

to the skin. These NO-induced CD25+Foxp3+CD127-Tregs had immunosuppressive functions and inhibited autologous CD4+ T cell proliferation. Cytokines, at least IL-10, secreted by NO-treated CD4+ T cells were not sufficient for the suppressive function of NO-induced Foxp3+Tregs. The immune regulatory function of NO-induced Fopx3+Tregs required cell-cell contact and was mediated by membrane bound TGFβ and PD-1/PD-L1 but not CTLA-4.

Results also showed that *cis*-UCA might have both pro- and anti-inflammatory effects. *Cis*-UCA significantly decreased the proportion of CD25hi Foxp3+ cells from activated CD4+ T cells. It also decreased the expression of vitamin D receptor in CD4+ T cells which may interfere with the immune regulatory function of vitamin D. These results suggested that there might be a fine balance between UV-induced anti-inflammatory molecules' effect on CD4+ T cells. However, *Cis*-UCA also modulated CD4+ T cell directly by decreasing CD4+ T cell proliferation, decreasing phosphorylation of ERK after TCR activation, enhancing immune suppressive cytokines secretion, and inhibiting the percentage of CLA+CD4+T cells suggesting a decreased ability to migrate to the skin, . *Cis*-UCA also affected the phenotype and function of antigen presenting cells by decreasing the expression of HLA-DR, CD86 and CD40 on immature mo-DCs, which led to increased proportion of CD25+Foxp3+CD127- T cells when co-cultured with allogenic CD4+ T cells.

Results generated from the clinical study in which all 29 patients got better after phototherapy suggested although circulating 25 (OH) vitamin D concentration was significantly increased after NB-UVB phototherapy, the change of circulating 25 (OH) vitamin D concentration did not correlate with disease improvement. This suggests that vitamin D is not the only pathway involved and that other molecules contribute to UVB-induced immune-regulation. The data

also show that of the levels of circulating nitrate and the FLG genotype did not correlate with improvement / change with phototherapy. However, the expression of CD69 and CLA on circulating CD4+ T cells was decreased after treatment suggesting that UVB affected T cell activation and migration to the skin, and their importance in determining clinical responses requires further investigation.

Taken together, the results from my study provide evidence that vitamin D is not the only molecule responsible for the beneficial effect of NB-UVB phototherapy. NO and *cis*-UCA may down-regulate immune responses by affecting human peripheral CD4+ T cells and mo-DCs phenotype and function. A further understanding of the effect of NO and *cis*-UCA on skin resident immune cells will provide more insights for narrowing NB-UVB phototherapy which will help to select patients that most likely to benefit from a mechanism-based treatment.

Abbreviations

AD	atopic dermatitis
AF700	AlexaFluor700
AMP	antimicrobial peptide
APC	antigen presenting cells
Br-cGMP	8-Bromoguanosine 3′,5′-cyclic monophosphate sodium salt
cDNA	Complementary DNA
cGMP	cyclic guanosine monophosphate
CHS	Contact hypersensitivity
CLA	cutaneous lymphocyte-associated antigen
CTLA-4	cytotoxic T cell associated antigen-4
CTLs	cytotoxic T lymphocytes
DC	dendritic cell
DTH	delayed type hypersensitivity
Ebi3	Epstein-Barr-virus-induced gene 3
ECL	enhanced chemiluminescence
FcεRI	high affinity receptor for IgE
GITR	glucocorticoid-induced TNF receptor family-related gene/protein
HDM	house dust mite
HNO_2	nitrous acid
ICAM-3	intercellular adhesion molecule-3
IDEC	inflammatory dendritic epidermal cells
IDO	Indoleamine 2, 3-dioxygenase

IFN-γ	interferon gamma
IL-2	Interleukin 2
IL-2R	Interleukin 2 Receptor
IL-12α	interleukin-12 alpha
IPEX	immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iTreg	induced regulatory T cell
LAP	latency-associated peptide
LC	Langerhans cell
L-NMMA	NG-monomethyl-L-arginine acetate
LAP	latency-associated peptide
MACS	magnetic activated cell sorting
MFI	mean fluoresce intensity
MLR	mixed lymphocyte reaction
Mo-DC	monocyte-derived dendritic cell
NB-UVB	Narrow band UVB
NK cells	natural killer cells
NMF	natural moisturizing factors
N-nitrosamine	RNNOs
NO	nitric oxide
NOC-18	3,3-Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene
nTreg	Natural regulatory T cell
ODQ	1-H-oxodiazolo-[1,2,4]- [4,3-a]quinoxalin-1-one
PAR	proteinase-activated receptor

PBMC	peripheral blood mononuclear cell
pCPA	4-Chloro-DL-phenylalanine
PD-1	Programmed death-1
Poly I/C	Polyinosinic-polycytidylic
PVDF	Polyvinyl difluoride
RANK	receptor activator of NF- κB
RANKL	receptor activator of NF- κB ligand
RIPA	Radio Immunoprecipitation Assay
sGC	Soluble guanylyl cyclase
RSNO	S-nitrosothiols
SPF	sun protection factor
TCR	T cell receptor
Th2	T helper type 2 cell
Th3	T helper type 3 cell
Treg	regulatory T cell
TLR	toll like receptor
Tr1	T helper type 1 cell
TSLP	thymic stromal lymphopoietin
UCA	urocanic acid
VCAM-1	vascular cell adhesion molecule-1
VDR	vitamin D receptor
vitD	1,25(OH)2D3

Chapter 1 Introduction

Atopic dermatitis (AD) is the most common chronic relapsing inflammatory skin disease associated with cutaneous hyper-reactivity to environmental triggers that are innocuous to non-atopic individuals (Leung et al., 2004). The majority of cases, at least 60%, arise within the first year of life (Bieber, 2012). The prevalence of AD has increased 2-3 times during past three decades and affects 10–20% in children and up to 5% in adults in industrialized countries (Boguniewicz and Leung, 2011). In the past few years, studies have shown that there is strong association between mental health and AD (Leung and Guttman-Yassky, 2014). Effective management of AD contributes to the well-being of patients and their family's quality of life. There has been increasing interest in this disease triggered by its increasing prevalence in western societies and its contribution to the increasing health care costs.

The symptoms of the majority of AD patients improve with first-line topical treatment including emollients, corticosteroids, calcineurin inhibitors and environmental trigger avoidance. Phototherapy is recommended in both acute and chronic AD after failure of first-line treatment. Phototherapy can also be used as maintenance therapy and is indicated for patients with chronic disease. Compared with other phototherapies, narrowband UVB therapy (NB-UVB) is generally the most commonly recommended light treatment considering its low risk profile, relative efficacy and availability (Eichenfield et al., 2014). However, in comparison with psoriasis, which is still the most frequent indication for NB-UVB phototherapy, a reasonable number of AD patients do not respond to the treatment. In general, patients with AD do less reliably respond to phototherapy. Therefore, it is important to identify the molecular mechanisms responsible for the beneficial effects of NB-UVB, and also to identify potential biomarkers that can be used to identify patients with AD that will

respond to phototherapy. These characteristics including genotype, biomarkers reflecting immune polarization and the clinical phenotype, can be used in further clinical study design and drug development to use existing or novel therapies to patients that most likely to benefit from a mechanism-based treatment.

1.1 Atopic dermatitis

1.1.1 Normal structure and immune responses in skin

Human skin as the primary interface between the body and the environment has two main compartments: the epidermis and the dermis as shown in Fig 1.1. The skin acts as an effective physical barrier against pathogens and exerts critical immunological functions during homeostasis and in various pathological conditions. The epidermis contains four layers including stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Besides keratinocytes and melanocytes, immune cells such as Langerhans cells (LCs) which are the major antigen presenting cells in epidermis and mainly CD8+ T cells can be found in the epidermis. Dermis is less densely packed with cells comp ared to epidermis and is composed of extracellular matrix produced by fibroblasts. Some important immune cells have been found in dermis including various dendritic cell (DC) subsets, CD4+ T cells, macrophages and mast cells.

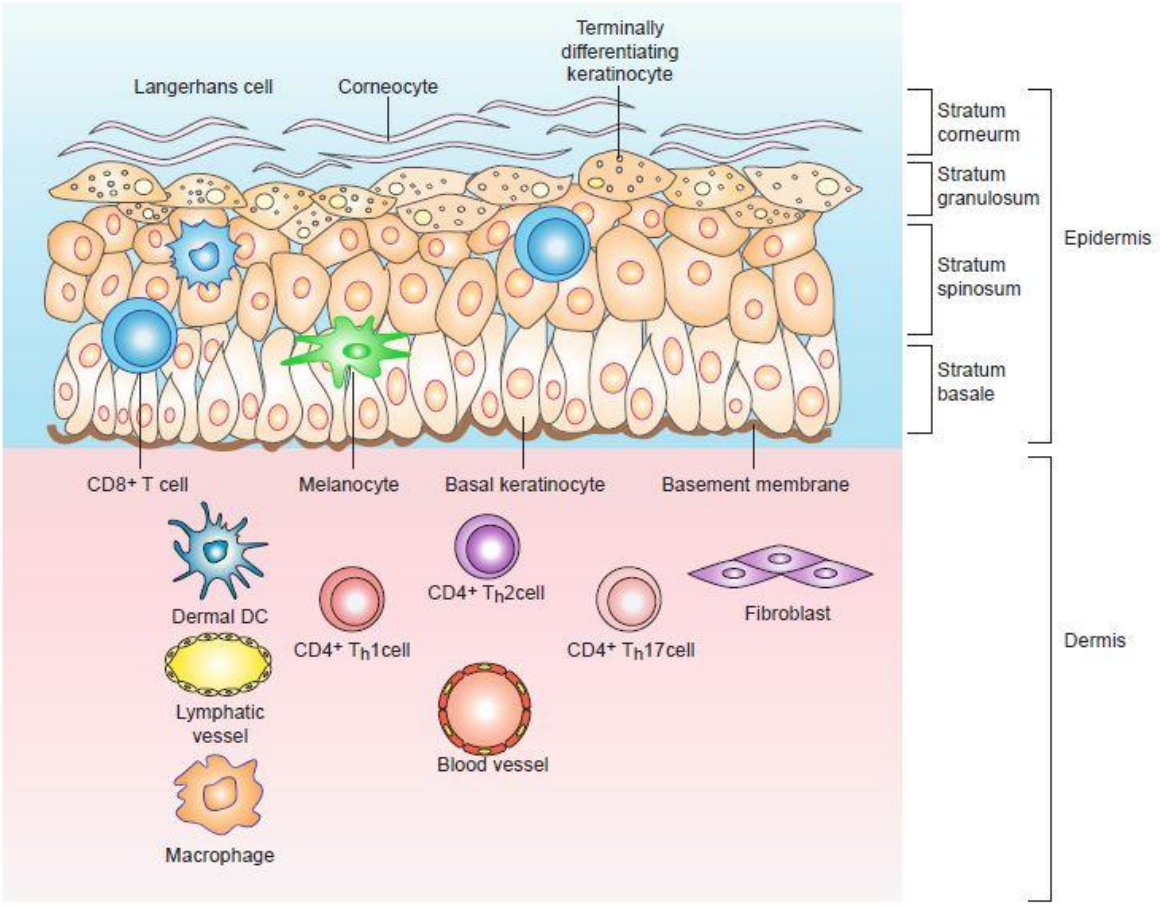


Fig 1.1 Normal skin structure and immune cell subsets in the epidermis and the dermis.

1.1.2 Clinical features and phenotypes of AD

AD used to be delineated as two forms: an extrinsic form associated with high IgE concentration involving 70–80% of the patients, and an intrinsic form without IgE-mediated sensitization involving 20–30% of the patients. However, it is reported that intrinsic patients had detectable serum IgE to autoantigens in the skin and may also have IgE specific to microbial or fungi antigens which are not routinely measured (Novak et al., 2003). Recent studies suggest that AD is not a single disease and should be categorized based on gene variations, immune polarization and natural history of the disease (Bieber, 2012) .

AD is a major risk factor for developing food allergy, allergic rhinitis, or asthma later in life (Spergel, 2010). Studies have shown that peanut sensitization caused by environmental exposure is increased in children with AD which indicates that the defective skin barrier caused by AD allows penetration of environmental allergens through the skin and promotes systemic allergen sensitization leading to the development of the so-called atopic march (Brough et al., 2015). Therefore, it is important to understand the underlying pathophysiologic and genetic mechanisms leading to the manifestation of AD.

1.1.3 Pathogenesis of AD

The etiology of AD involves a complex interplay between environmental triggers, susceptibility genes and altered immune responses (Elias et al., 2008).

1.1.3.1 Environmental factors

Studies have shown that microbial antigens can exacerbate AD. Comparing with 5-10% of healthy individuals who carry Staphylococcus aureus, 90% of AD patients are colonized with this microorganism (Savinko et al., 2005). There is increasing evidence show that *S. aureus* secreted superantigen can penetrate into the dermis and triggers cutaneous inflammation. Even low dose of superantigen secreted by *S. aureus* can amplify aeroallergen-induced responses in patch tests (Werfel, 2009).

Clinical studies also suggest that contact with aeroallergens is important in determining disease expression. House dust mite (HDM) can be considered as a predominant provider of aeroallergens within the world, which could produce more than 20 different allergen groups

classified according to their sequence homology and biological function (Thomas et al., 2002). The degree of sensitization to aeroallergens is directly associated with the severity of AD. A previous study found that more than 80% of AD patients had IgE specific to HDM in their serum compared with 42% of asthmatic subjects (Boguniewicz and Leung, 2011). Atopic individuals have higher number of circulating HDM-specific T cells than non-atopic controls (Nurse et al., 2000). T cells isolated from AD lesional skin and allergen patch test sites recognize HDM and other aeroallergens, providing evidence that inhalant allergens could exacerbate the inflammatory response in AD (Leung and Bieber, 2003).

1.1.3.2 Multi-functional role of filaggrin

Recent advances in the genetics and pathophysiology of AD contribute to our understanding of the etiology of AD. Dry skin and increased trans-epidermal water loss are hallmarks of AD, which indicate an impairment of skin barrier function. Decreased skin hydration alone is able to stimulate epidermal hyperplasia and early evidence of inflammation, such as mast cell degranulation, even in normal skin. There is increasing evidence showing that a combination of genetic and acquired factors contribute to reduced epidermal differentiation and down-regulation of epidermal barrier function. In a search for susceptibility loci for AD, a number of gene variants have been linked to AD such as genes encoding a cluster of proteins in the epidermal differentiation complex located on chromosome 1q21 which includes filaggrin 2, hornerin, and the cornified envelope precursor SPRR3 (Marenholz et al., 2011). Aside from the epidermal differentiation complex, loss-of-function mutations in serine protease inhibitors such as SPINK5 have been suggested to contribute to AD by enhancing protease-activated pathways that promote Th2 differentiation such as increasing the secretion of proallergic

cytokine, thymic stromal lymphopoietin (TSLP) (Cork et al., 2009).

To date, the strongest evidence for a primary structural abnormality contributing to the pathogenesis of AD is loss of function or missense mutations in the gene FLG encoding filaggrin (filament-aggregating protein), a protein that is important for epidermal differentiation, desquamation and barrier function. Copy number variation of FLG genes contributes to the risk of AD as well (Palmer et al., 2006). A recent study showed that epigenetic modification leading to reduced expression of FLG also significantly increased the risk of AD (Ziyab et al., 2013). The 'outside-inside' hypothesis suggests that filaggrin deficiency in AD contributes to a leaky skin epithelial barrier which allows enhanced aeroallergen and microbe penetration resulting in epicutaneous sensitization and decreased hydration of the stratum corneum with increased trans-epidermal water loss (Elias et al., 2008).

Filaggrin is a major structural component of the cornified envelope, which is synthesized as a large precursor, profilaggrin, stored in the keratohyalin granules in the granular layer. During the transit from the granular cell layer to coenocyte, filaggrin is generated by proteolysis of profilaggrin mediated by enzymes such as furin, endoproteinase 1, calpain 1, matriptase, and elastase and released to aggregate keratin intermediate filaments into macrofibrils (Thyssen and Kezic, 2014). During the final cornification steps, filaggrin is proteolytically degraded into its constituent amino acids by caspase-14, calpain 1, and bleomycin hydrolase, which includes glutamine, arginine, and histidine, as well as their downstream deiminated metabolites. Histidine, which constitutes about 10% of the amino acids in filaggrin, is converted to trans-urocanic acid (trans-UCA) by histidase and glutamine, and further converted into pyrolidine carboxylic acid (PCA). Trans-UCA and PCA are the main components of the natural moisturizing factors (NMFs) of the skin accounting for corneocyte

hydration and pH (O'Regan et al., 2009) (Fig 1.2).

Depending on the population, FLG mutations are found in 25% to 50% of patients with severe AD (Weidinger et al., 2006). Low filaggrin expression is also found in AD patients with no detectable FLG-null mutations indicating that the levels of filaggrin expression are also modulated by other factors than the filaggrin genotype. Indeed, multiple causes contribute to the low expression of FLG in skin. A variety of cytokines including IL-4 and IL-13 have been shown to inhibit expression of filaggrin leading to the outside-inside-outside pathogenic loop in AD (Howell et al., 2007). Exposure to low ambient humidity and skin irritants enhances filaggrin proteolysis (Katagiri et al., 2003).

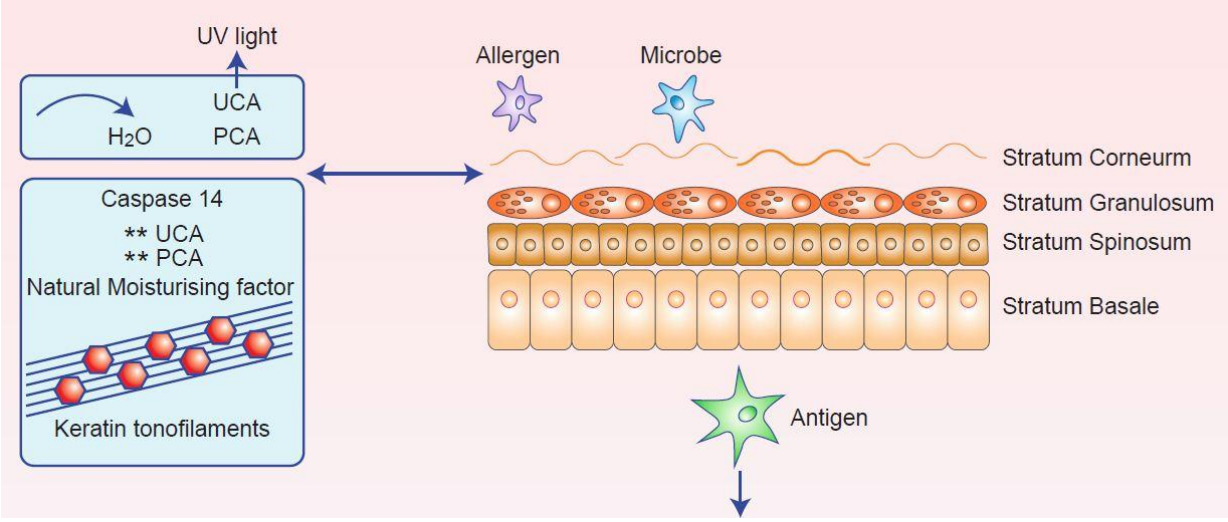


Fig1.2 Filaggrin breakdown in the epidermis. Filaggrin is proteolytically degraded into its constituent amino acids by enzymes such as caspase-14. Histidine further converts to trans-UCA and PCA, which are the main components of the natural moisturizing factors. Trans-UCA converts to *cis*-UCA on exposure to UV irradiation.

AD patients with filaggrin null mutations compared with patients with normal filaggrin expression have early onset of disease, a more persistent course and a higher risk of IgE mediated sensitization. Stratum corneum pH is increased in patients with FLG mutations,

which might contribute to their 7-fold increase in the risk of *S aureus* infections (Cai et al., 2012). AD patients with FLG mutations have decreased expression of sphingomyelinase, which has been found to protect keratinocytes by reducing staphylococcal α-toxin binding (Brauweiler et al., 2013). Increased expression of IL-1 cytokines and stress responses mediated by type 1 interferon in the stratum corneum have been reported in patients with filaggrin null mutations (IRVINE, 2014). A positive association between FLG null mutations and allergen-specific CD4+Th2 response has also been reported (McPherson et al., 2010).

Filaggrin deficiency provokes a defective antimicrobial barrier and reduces the inflammatory thresholds to topical irritants (Elias and Schmuth, 2009). Filaggrin-deficient mice develop eczema as expected after skin exposure of allergens or microbes (Oyoshi et al., 2009). Filaggrin deficiency has also been shown to affect keratinocyte architecture. In flaky tail mice which is profilaggrin deficient, the expression of E-cadherin and occludin is reduced as well as the expression of growth factor receptors (Nakai et al., 2012). Similar results have been reported in AD patients with FLG mutations (Gruber et al., 2011). The breakdown products of filaggrin acidify the stratum corneum (SC) and decreased filaggrin metabolites increase the pH of SC leading to activation of several kinds of serine proteases. Activation of proteinase-activated receptors (PAR)-2 by endogenous and exogenous proteases delays epithelial regeneration and further compromises skin barrier function (Cork et al., 2009). Proteases also break down corneodesmosomes leading to compromised intercellular connections (Cork et al., 2009). Recent studies have shown that AD might be prevented by early application of emollient to protect the skin barrier (Simpson et al., 2014). Taken together, these evidence suggest that filaggrin plays an important role in the pathogenesis of AD.

1.2 Regulatory T cell differentiation and function

DCs take up antigen and travel from the site of infection into the draining lymph nodes, where they activate antigen-specific T lymphocytes. The activated T lymphocytes then undergo proliferation and differentiation into effector cells. Different helper T cell populations are defined by their unique patterns of cytokine secretion (Fig 1.3). Th1 cells are characterized by production of IFN- γ and responsible for clearing intracellular pathogens whereas Th2 cells produce IL-4, IL-5, and IL-13 and are key players for clearing extracellular pathogens and in helping B cells to produce antibodies. Th17 cells produce IL-17 and IL-22, which are important in regulating antimicrobial peptides production in keratinocytes. Th22 cells are defined by the production of IL-22, which induces epidermal hyperplasia and inhibit terminal differentiation. Regulatory T cells (Tregs) are important in maintaining self-tolerance and immune homeostasis. Therefore, their dysfunction can lead to chronic inflammation as seen in severe autoimmune diseases, and allergy.

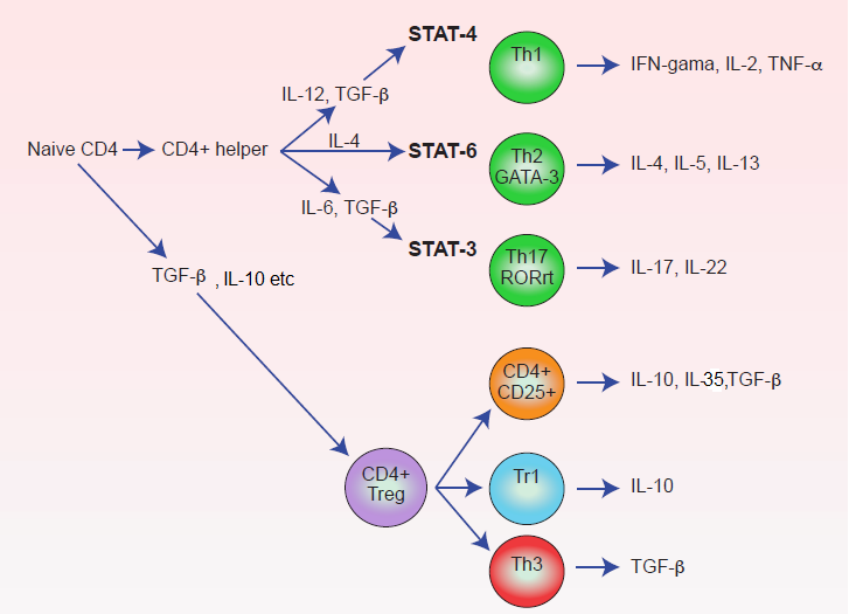


Fig 1.3 Different helper T cell populations are defined by their unique patterns of cytokine secretion.

1.2.1 Types of Tregs

Tregs are key to maintain self-tolerance and immune homeostasis. Tregs inhibit the activation, proliferation and effector function of several immune cells including T cells, B cells, NK cells and antigen presenting cells. Therefore, their dysfunction can lead to chronic inflammation as seen in severe autoimmune disease and allergy. Tregs can be divided into two major groups: the thymus-derived natural Tregs and adaptive Tregs that derived extra-thymically.

1.2.1.1 Thymus-derived natural Tregs (nTregs)

Thymus-derived CD4+CD25+ T cells are considered to be natural Tregs (nTregs), the development of which involves T cell receptor (TCR) activation by self-peptide-MHC complexes presented by thymic stromal cells (Maloy and Powrie, 2001). They are capable of recognizing diverse self-antigens and prevent the activation of self-reactive T cells developing into effector T cells in the periphery (Bluestone and Abbas, 2003).

1.2.1.2 Adaptive (Induced) Tres (iTregs)

Adaptive Tregs develop as a consequence of activation of naive T cells under particular conditions of sub-optimal antigen exposure and/or co-stimulation.

1.2.1.2.1 Foxp3+ iTregs

Foxp3+Tregs can be induced from peripheral naïve CD4 T cells in several experimental settings. *In vitro* experiments have shown that stimulation of naïve T cells in the presence of TGF-β promotes Foxp3+Treg differentiation. IL-2 promotes differentiation of murine naïve T cells to Foxp3+Tregs induced by TGF-β while it inhibits their differentiation to inflammatory Th17 cells induced by TGF-β and IL-6 (Laurence et al., 2007). Retinoic acid, secreted by

DCs in the gut-associated lymphoid tissue, facilitates the differentiation of naive T cells to Foxp3+ Tregs in the presence of TGF-β (Benson et al., 2007).

1.2.1.2.2 Type 1 regulatory T cells (Tr1)

Besides Foxp3+ iTregs, other types of Tregs can be induced from naïve T cells in the periphery. Type 1 regulatory T cells (Tr1) can be generated by stimulating naïve T cells in the presence of the suppressive cytokine IL-10 (Buckner and Ziegler, 2004). These Tr1 cells then exert their regulatory function by secreting IL-10 and TGF-β. It is reported by Kemper et al that human Tr1 cells can be generated by stimulating CD4+ T cells by co-ligation of CD3 and the complement regulator CD46 in the presence of IL-2, which exhibited strong and prolonged proliferation when stimulated (Kemper et al., 2003). Tr1 cells have been shown to control immune responses both *in vitro* and *in vivo*. Once Tr1 cells are activated through their TCR, their cytokine secretion means that they can exert bystander suppressive activity against T effector cells that recognize other antigens (Allan et al., 2008).

1.2.1.2.3 T helper 3 cells (Th3)

It has long been recognized that oral tolerance induces the generation of antigen-specific regulatory T cells known as Th3 cells (Weiner HL et al., 2001). Th3 cells produce TGFβ but no IL-10, which mediates the suppression of Th3 in *in vivo* transfer tolerance murine models. Reduced number of peripheral Tregs was found in TGFβ1 deficient mice. Mice, which overexpressed a dominant negative TGFβ II receptor on T cells leading to the lack of ability to respond to TGFβ, develop a fatal autoimmune lymphoproliferative disease, which suggests the importance of Th3 in preventing autoimmunity (Buckner and Ziegler, 2004).

1.2.2 Signals which drive Tregs differentiation

Foxp3 is a forkhead box transcription factor and the master regulator for Treg differentiation and function, as evidenced in scurfy mice and in patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. Although human CD4 effector T cells transiently express Foxp3 after activation, stable and high expression of Foxp3 is required for suppressive function of Tregs. Foxp3 transduction in CD4+CD25- T cells converts them to functional CD4+CD25+ Tregs, which also leads to the expression of other Treg associated surface molecules, such as cytotoxic T cell associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene/protein (GITR) (Hori et al., 2003). These results imply that high level of Foxp3 expression is able to induce suppressive function in T cells. Besides transcription factors, genome-wide studies have shown that nearly 700 genes can be modulated by Foxp3 directly or indirectly including molecules involving signal transduction, cytokines, cell metabolism related enzymes. (Marson et al., 2007)

Another molecule that is important for the differentiation and function of Tregs is Interleukin 2 (IL-2). CD25, which is an important marker for Tregs, is a component of the high affinity IL-2 receptor (IL-2R). The crucial role of IL-2 in Treg function has been demonstrated in mice lacking IL-2, which spontaneously develop T cell-mediated fatal lymphoproliferative/ inflammatory disease and have decreased number of Foxp3+Tregs (Antony et al., 2006). Neutralizing IL-2 with monoclonal antibody in normal neonatal mice decreased the number of CD4+CD25+Foxp3+ Tregs and lead to the elicitation of autoimmune diseases (Setoguchi et al., 2005). *In vitro* experiments have shown that sustained expression of CD25 and Foxp3 require IL-2, which also enhances the suppressive ability of Tregs (Fontenot et al., 2005). Therefore, a negative feedback loop exists between activated T effector cells and Tregs, that is, activated T cells produce IL-2 which will maintain, expand and activate Tregs.

1.2.3 Mechanisms of Treg-mediated suppression

Several mechanisms have been proposed to mediate suppressive function of regulatory T cells.

1.2.3.1 Suppression by inhibitory cytokines

Interleukin‑10 (IL-10) and TGFβ have been suggested as mediators of Tregs induced immunosuppression. For example, IL-10 produced by UV-induced Tregs mediates the anti-tumour responses in a UV-radiation-induced murine model of carcinogenesis (Loser et al., 2007). Neutralising IL-10 and/or TGF-β reverses the suppression of the proliferative allo-response induced by Tr1 cells. IL-10 secreting Tr1 have also been shown to suppress antibody production of B cells and antigen presenting ability of monocytes and dendritic cells (Buckner and Ziegler, 2004). The importance of IL-10 produced by Tregs as a mechanism of Treg mediated suppression depends on the target and the disease model. In murine inflammatory bowel disease induced by Treg depletion, IL-10 produced by Tregs have been shown to be essential in suppression of colitis while in a murine allergy and asthma model, suppression of disease only partially depends on IL-10 (Vignali et al., 2008).

TGFβ produced by Tregs has been reported to suppress inflammation by rendering responder T cells sensitive to suppression and/or maintaining Foxp3 expression and suppressive activity in Tregs (Sakaguchi et al., 2008). TGFβ has also been suggested to promote the development of IL-10 secreting Tregs, as neutralizing TGFβ inhibits the conversion of CD4+Foxp3- effector T cell to CD4+Foxp3+IL-10 secreting Tregs in intestine-associated lymphoid tissues (Shevach, 2009). Although some *in vitro* studies showed that neutralizing TGFβ did not reverse Treg mediated suppression, it is suggested by Nakamura *et al* that TGFβ produced by Tregs binds to the cell surface and mediates suppression in a cell contact dependent fashion. The regulatory activity of TGFβ is affected by several factors such as the differentiation states of target cells and the presence of additional regulatory signals, including costimulatory

molecules and inflammatory cytokines. For example, TGFβ inhibits naïve T cell proliferation by suppressing the expression of IL-2 while it has minimal effect on activated T cells which have reduced expression of TGFβRII expression (Li et al., 2006).

Besides IL-10 and TGFβ, IL-35 which belongs to the IL-12 heterodimeric cytokine family is described to be expressed by Tregs and is required for their suppressive function (Vignali et al., 2008). IL-35 is formed by Epstein-Barr-virus-induced gene 3 (*Ebi3*, which encodes IL-27β) and interleukin-12 alpha (*Il12α*, which encodes IL-12α/p35). Cell contact between suppressor and responder cells induces Ebi3 and Il12α mRNA expression in Tregs leading to the maximal IL-35 production (Shevach, 2009). Recombinant IL-35 suppresses T-cell proliferation *in vitro* while Tregs from Ebi3-/- and IL-12α-/- knockout mice have decreased suppressive function and cannot control inflammatory bowel disease *in vivo*. (Collison et al., 2007).

1.2.3.2 Suppression by cytolysis

Granzymes contain a family of serine proteases and are stored within cytoplasmic granules of CD8 cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Granzymes induce cell apoptosis by cleaving and activating intracellular caspases. Gondek C *et al* suggest that Granzyme B is one of the key mechanisms which mediate the cell contact dependent suppression of Tregs by showing that Tregs from GZ-B-/- mice have reduced suppressive ability compared to Tregs from wild type mice (Gondek et al., 2005). Adaptive Tregs, which are generated by stimulating CD4 T cells with CD3 and CD46 in the presence of IL-2, express granzyme B and kill autologous target cells in a granzyme B and perforin dependent manner (Grossman et al., 2004). The target cells for the granzyme B mediated cytolytic function of Tregs include CD4 T cells, B cells, NK cells, CTLs, DCs (Vignali et al., 2008).

1.2.3.3 Suppression by targeting DCs

Tregs are proposed to induce expression of immunosuppressive molecules in DCs. Co-stimulatory molecule cytotoxic T‑lymphocyte antigen 4 (CTLA-4) is constitutively expressed by Tregs. The interaction between CTLA-4 and CD80/CD86 on DCs resulted in Indoleamine 2, 3-dioxygenase (IDO) expression, which catalyses tryptophan metabolism and leads to the production of pro-apoptotic metabolites (Fallarino et al., 2003).

Tregs are proposed to modulate the maturation and function of DCs, which in return modulate the activation of effector T cells. Ilona Kryczek *et al* reported that Tregs induced IL-10 secretion from antigen presenting cells (APCs) and promoted the expression of B7-H4 on APCs via IL-10, which renders APCs immunosuppressive (Kryczek et al., 2006). Namita Misra *et al* suggested that human mo-DCs co-cultured with autologous CD4+CD25+ Tregs had reduced level of co-stimulatory molecules expression including CD86, CD40 and HLA-DR and increased IL-10 secretion (Misra et al., 2004).

1.2.3.4 Suppression by cell-contact dependent effect

1.2.3.4.1 Membrane bound TGFβ

Both *in vitro* and *in vivo* studies have proved that membrane-bound TGFβ is critical for nTreg function (Gandhi et al., 2010). Following a post-translational maturation, the amino terminal domain of TGFβ binds non-covalently with latency-associated peptide (LAP), which is a pro-peptide, forming an inactive form of TGFβ. Nakamura *et al* showed that mouse CD4+LAP+ Tregs exerted their suppressive function via a TGFβ–dependent manner and were effective in suppressing autoimmunity in a murine model of multiple sclerosis (Nakamura et al., 2004).

Several cell surface proteins have been reported to be involved in the activation of membrane-bound TGFβ, which includes thrombospondin-1, CD36 on macrophages and CD47 on T cells (Nakamura et al., 2001). TGFβ initiates its effects on cells through the

binding of TGFβ to a heteromeric complex, which comprises TGFβ type I (RI) and type II (RII) receptors. Ligand binding leads to phosphorylation of downstream signalling proteins including SMAD2 and SMAD3, which then translocate into the nucleus and modulate transcription directly or indirectly by binding with other transcription factors (Fontenot et al., 2003).

Chen *et al* showed that responder cells up-regulate the expression of TGFβ receptor type II after being activated through TCR, which provided the opportunity for suppressor derived TGFβ to bind to activated responder cells and transduce suppressive signals via contact-dependent manner, as evidenced by the increased phosphorylation of SMAD2/3 in responder cells upon contact with suppressor cells (Chen and Wahl, 2003)

1.2.3.4.2 CTLA-4

Human T cells express HLA-DR and co-stimulatory molecules upon activation. Therefore, costimulatory pathways provide a mechanism to promote the development and function of Tregs in the absence of APC as well as control the fate of naive T cells upon antigen encounter. CTLA-4 is constitutively expressed on Tregs (Fife and Bluestone, 2008). Active form of membrane bound TGFβ and TGFβ receptor type II expression on CD4+CD25+ suppressor T cells were up-regulated by CTLA-4 ligation. CTLA-4-B7 interactions have also been shown to occur directly between Treg-Teff, which contributes to the suppression of Teff function (Paust and Cantor, 2005). CTLA-4 delivers a negative signal antagonizing early T cell activation by inhibiting IL-2 synthesis and cell cycle progression (Fife and Bluestone, 2008).

1.2.3.4.3 Programmed death-1(PD-l)

Besides CTLA-4, PD-1 is another key negative regulatory molecule that critically affects

peripheral T-cell function. Gene expression profiling of CD4 T cell co-stimulated with PD-1 and CTLA-4 showed that PD-1 has stronger inhibitory effect than CTLA-4 on T cell activation (Francisco et al., 2010). PD-1 is induced in a variety of cells in the periphery upon activation including CD4+ and CD8+ T cells, NK T cells, B cells, monocytes, and some DC subsets (Francisco et al., 2010).

PD-1 interacts primarily with two ligands: programmed death ligand-1 (PD-L1), which is expressed on leukocytes, non-hematopoietic cells and in non-lymphoid tissues and PD-L2, which is expressed exclusively on DCs and monocytes. Signalling through PD-1 limits T-cell function including cytokine production, proliferation and cytolytic function. PD-1 signalling also decreases T cell effector function related transcription factors including GATA-3 and T-bet (Riella et al., 2012). The extent of PD-1 mediated inhibition is influenced by strong TCR stimulation. PD-1 suppresses T-cell function and survival directly by inhibiting early activation signals induced by CD28 or indirectly through IL-2, therefore, CD28 co-stimulation and IL-2 can reverse PD-1 mediated suppression (Francisco et al., 2010).

Studies have shown that PD-L1 is also involved in iTreg generation. Francisco LM *et al* reported that co-stimulation with PD-L1 promotes the generation of Foxp3+iTregs. The expression of Foxp3 and suppressive function of Foxp3+ iTregs were also enhanced by PD-L1 co-stimulation (Francisco et al., 2009). Since both nTregs and iTregs express PD-L1, they may further assist the generation of iTregs. PD-L1 expressed on iTregs may engage PD-1 on dendritic cells and modulate DC function, which indirectly suppress effector T cells (Yao et al., 2009).

1.3 Altered T cell responses in AD

1.3.1 Th2 cells in AD

Infiltration of large numbers of T cells is considered to be one of the key features in the pathogenesis of AD. In acute lesional AD skin, there are increased levels of Th2 cells and associated cytokines (IL-4, IL-5 and IL-13), which are correlated with disease severity. In non-lesional AD skin, a selective expansion of Th2 cells in the dermal perivascular region is reported (Guttman-Yassky et al., 2011). IL-4 and IL-13 play a critical role in differentiation of allergen-specific Th2 cells and in immunoglobulin isotype switching to IgE leading to the initiation of allergic inflammation. The expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells is enhanced by Th2 responses as well, which leads to increased inflammatory cell infiltration (Guttman-Yassky et al., 2011). The Th2 dominant microenvironment has profound impact on skin integrity by down-regulating the expression of filaggrin, loricrin, and involucrin and the production of anti-microbial peptide (AMP) which contributes to the overabundance of *S. aureus* (Howell et al., 2007). IL-5 is suggested to be involved in eosinophil survival while another Th2 cytokine IL-31 has been shown to increase pruritus (Raap et al., 2012). AD patients with filaggrin null mutations have increased level of circulating allergen-specific Th2 cells, which supports the 'outside-inside' hypothesis (McPherson et al., 2010).

1.3.2 Th1 cells in AD

AD is suggested to be a biphasic inflammation and the chronic phase used to be considered as Th1-dominated but not a complete switch to Th1 (Gittler et al., 2013). IL-12, a key cytokine

of Th1 polarization is reported to be down-regulated in acute skin lesions in AD, which is detected at mRNA level by in situ hybridization (Werfel, 2009). IFNγ, a characteristic Th1 type cytokine, upregulated Fas expression on keratinocytes which binded with Fas ligand expressed on activated T cells leading to keratinocyte apoptosis (Goyette and Geczy, 2011). Downregulation of IFNγ but not IL-4 in the skin correlates with disease improvement after treatment(Werfel, 2009).

1.3.3 Th17 cells in AD

A few studies have investigated the role of IL-17-secreting Th17 cells in the pathogenesis of AD. Immunohistochemistry studies have showed that Th17 axis is preferentially increased in acute AD skin lesions and circulation while it is reduced in chronic lesions (Koga et al., 2008). In acute lesions, increased production of IL-17 is induced by Staphylococcal enterotoxin B, which may directly contribute to the acute phase of AD by inducing B cells to produce antigen-specific IgE (Toda et al., 2003; Milovanovic et al., 2010). It has been shown in a murine model that filaggrin deficiency predisposes Th17-dominated skin inflammation, the severity of which is positively correlated with IL-17 expression in epidermis (Oyoshi et al., 2009). IL-17 has been shown to induce AMP production in human keratinocytes both *in vitro* and *in vivo* although this effect is inhibited by Th2 cytokines leading to increased susceptibility to bacterial infection in AD (Novak and Leung, 2011).

1.3.4 Th22 cells in AD

A recently discovered T cell subpopulation T22, which contains CD4+ (Th22) and CD8+

(Tc22) cells has been found to be increased in AD, the frequency of which is associated with AD severity (Eyerich et al., 2009). IL-22 affects keratinocyte function by inhibiting terminal differentiation and increasing proliferation leading to epidermal hyperplasia (Nograles et al., 2009). Enhanced epicutaneous antigen penetration induced by defective barrier in AD is process by LCs in epidermis, which will activate Th2 and Th22 immune responses and promote the aggravation of AD (Fujita et al., 2009). IL-22 production can also be induced by staphylococcal enterotoxin B (Niebuhr et al., 2010). IL-22 has been reported to affect the expression of pro-filaggrin processing enzyme leading to downregulation of filaggrin, thus further disrupting epidermal barrier function in AD (Gutowska‐Owsiak et al., 2011).

1.3.5. Tregs in patients with AD

Tregs play a vital role in the resolution of allergic immunity and increasing their number and/or function may offer benefit to AD patients. Controversial results have been published about the number and function of Treg cells in AD patients. , as further described below. Not all the studies actually identified Tregs in the same way and this might lead to some of the controversy. Table I summarizes the phenotype used to define human Tregs.

Table 1.1 Summary of phenotypes used to define Tregs in human.

Phenotype of human Tregs	Reference
CD4+CD25+ Tregs	(Dieckmann et al., 2001)
CD4+CD25hi Tregs	(Baecher-Allan et al., 2005)
CD4+CD25+Foxp3+ Tregs	(Walker et al., 2003)
CD4+CD25hiFoxp3+ Tregs	(Ahmadzadeh and Rosenberg, 2006)
CD4+CD25+Foxp3+CD127lo/- Tregs	(Liu et al., 2006)
CD4+CD25+ CD127lo/- Tregs	(Hartigan-O'Connor et al., 2007)

1.3.5.1 Studies reporting decreased number and function of Tregs in patients with AD

Some studies demonstrate decreased frequency and function of Tregs in AD patients. Stelmaszczyk-Emmel *et al* investigated the role of CD4+CD25 (+/high) FoxP3+ CD127- Tregs in 20 allergic patients among whom 10 had AD. Compared with healthy controls, a significantly decreased frequency of Tregs was observed in all patients, which negatively correlated with concentration of allergen-specific IgE (Stelmaszczyk-Emmel et al., 2013). This observation was confirmed by Ma *et al* who showed that the percentage of CD4+ CD25high Foxp3+ Treg was decreased in peripheral blood and in cell suspensions obtained from skin samples obtained from patients with AD. Besides CD4+ CD25high Foxp3+ Tregs, serum concentration of the immune suppressive cytokine TGFβ was decreased as well as its mRNA expression in PBMCs from patients with AD (Ma et al., 2014).

Immunohistochemistry showed absence of CD25+Foxp3+ Treg in skin lesions from AD patients (Verhagen et al., 2006). Defective regulatory activity of CD25+ Tregs has been reported in two studies and their deficiency were associated with the atopic status of AD patients (Zhang et al., 2015; Ling et al., 2004).

1.3.5.2 Studies reporting similar number and function of Tregs in patients with AD

On the other hand, several studies report that AD patients have similar number of Tregs as healthy individuals. Vukmanovic-Stejic, *et al* reported that there was no difference in frequency of CD4+CD25+ T cells in peripheral blood of AD patients compared with healthy controls. CD4+CD25+ T cells isolated from AD patients inhibited proliferation of CD4+CD25- cells induced by purified protein derivative or Derp1 to a similar extend as those from healthy controls (Vukmanovic‐Stejic et al., 2005). It has been shown that CD4+CD25+ T cells from the majority of AD patients who are allergic to grass or birch pollen exhibit normal suppressive function by inhibiting proliferation and cytokine production of Th1 and Th2 cells. No difference in the frequency of CD4+CD25+ T cells was observed in these patients as well (Bellinghausen et al., 2003).

1.3.5.3 Studies reporting increased number and function of Tregs in patients with AD

Moreover, there are also studies suggesting that the frequency of Tregs is increased in AD. Several studies have shown that patients with AD have increased numbers of Foxp3+Tregs in peripheral blood compared to healthy control, and this is positively correlated with their AD severity (Reefer et al., 2008; Ou et al., 2004; Ito et al., 2009; Lesiak et al., 2012; Samochocki

et al., 2012). Treatments that improve skin lesions such as cyclosporin A significantly decrease Foxp3+Tregs frequency in peripheral blood and expression of Foxp3 in CD4 T cells at mRNA level (Hijnen et al., 2009).

Fujimura *et al* also reported that the epidermis and dermis of AD skin contain substantial number of CD25+Foxp3+ Tregs while in normal skin Foxp3+Tregs are rare (Fujimura et al., 2008). This observation is confirmed by Caproni M *et al* who showed that increased numbers of CD25+ Foxp3+ cells are distributed in the perivascular, interstitial, and periadnexal dermis compared with healthy skin (Caproni et al., 2007).

Although the studies mentioned above report increased frequency of Foxp3+Tregs, controversial results about function of Foxp3+Tregs in these studies have been shown. Samochocki *et al* suggest that CD4+CD25hi Foxp3+ cells from severe AD patients may have enhanced suppressive function as evidenced by increased expression of CD62L and CD134 and decreased expression of apoptotic CD95 receptor (Samochocki et al., 2012). Ou *et al* investigate function of CD4+CD25+ from patients with AD and non-atopic healthy control subjects by co-culturing them with autologous CD4+CD25- effector cells stimulated by anti-CD3 or staphylococcal enterotoxin B. They demonstrate that proliferation of effector cells is equally suppressed by CD4+CD25+ cells from patients with AD or controls when effector cells are activated by anti-CD3 while the suppressive function of CD4+CD25+ cells is lost when effector cells are stimulated by staphylococcal enterotoxin B (Ou et al., 2004). It is also suggested that skin-homing CD4+Foxp3+ Tregs are heterogeneous, which could be further divided into two subtypes based on expression of the chemokine receptor CCR6. CCR6- cells within skin-homing CD4+Foxp3+ Tregs produce the Th2 cytokine IL-5 after Staphylococcal enterotoxin B stimulation while the CCR6+ subtype produces the anti-inflammatory cytokine IL-10. (Lin et al., 2011). Taken together, these studies suggest that frequency and function of

Tregs may be differentially affected and it is important to test the function of cells with Tregs phenotype.

The presence of IL-10 secreting Type 1 regulatory T cells (Tr1) have been investigated as well. A Szegedi *et al* show that the number of Tr1 is significantly increased in peripheral blood of AD patients (Szegedi et al., 2009). The presence of Tr1 in AD skin has been confirmed by Verhagen et al who also show that Tr1 related cytokines including IL-10 and TGFβ and their receptors are abundantly expressed in AD skin (Verhagen et al., 2006). Taken together, these data suggest that one of the reasons contributing to controversial results of Tregs in AD patients may be that different markers are used to identify Tregs in humans.

1.4 Narrowband UVB Phototherapy

The UV wavelengths are divided further into three ranges as shown in Fig 1.4: UVA (320-400 nm), UVB (290-320 nm), and UVC (100-290 nm). The UVA spectrum has been subsequently divided up into UVA-1 (340-400 nm) and UVA-2 (320-340 nm). Within solar irradiation, UVC radiation and majority of UVB radiation are blocked by the ozone layer in the stratosphere and does not reach the earth's surface. Phototherapy uses a specific wavelength of UV radiation relying on controlled artificial light sources to treat a variety of skin diseases. The symptoms of the majority of AD patients improve with first-line topical treatment including emollients, corticosteroids and calcineurin inhibitors and environmental trigger avoidance. Phototherapy is recommended in both acute and chronic AD after failure of first-line treatment. Phototherapy can also be used as maintenance therapy and is indicated for patients with chronic disease. Narrowband UVB (NB-UVB) therapy uses a fluorescent bulbs emitting a major peak at 311 (±2 nm) and a minor peak at 305 nm. Compared with other phototherapy, NB-UVB is generally the most commonly recommended light treatment

considering its low risk profile, relative efficacy and availability (Eichenfield et al., 2014)

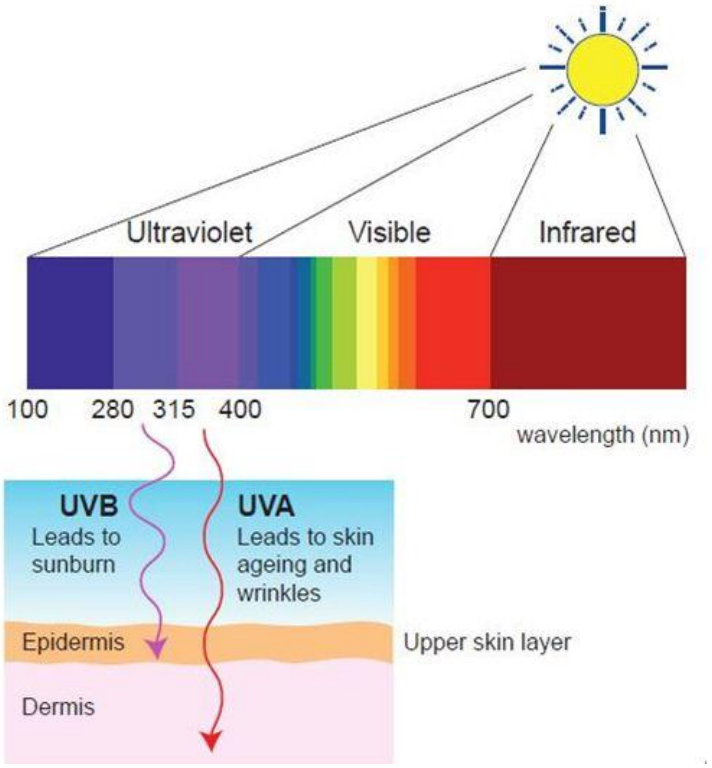


Fig 1.4 Spectrum of Sunlight and damage caused by different UV wavelength.

1.4.1 Mechanisms of NB-UVB phototherapy in treating AD

The goals of phototherapy are to suppress on-going inflammatory processes and to prevent or modulate the pathogenic mechanisms causing the disease. NB-UVB phototherapy has proved effective in treating AD and other inflammatory skin diseases, however, detailed understanding of the mechanisms underlying phototherapy remain elusive. As the biological effects of UVB are the best understood, the mechanisms summarised below will focus on the mechanisms induced by UVB in improving skin disease.

1.4.1.1 UV-induced apoptosis

Considering the important role of T cells in the pathogenesis of AD, the therapeutic effect of UVB is mediated partially by depleting effector T cells in skin. *In vitro* experiments have shown direct toxic effect of UVB on pure T cell populations. Besides direct toxicity, induction of apoptosis by UVB is the primary mechanism of depleting skin-infiltrating T cells (Weichenthal and Schwarz, 2005). Studies have shown that NB-UVB phototherapy is superior compared to broadband UVB phototherapy in depleting T cells from skin lesions. (Novák et al., 2002). It has been reported that several pathways, which act in an additive manner, are involved in UVB induced apoptosis including UVB-induced DNA damage (cyclobutane pyrimidine dimers and (6–4) photoproducts) and direct activation of death receptors ligand such as CD95Lon keratinocytes leading to neighbouring lesional T cell apoptosis (Weichenthal and Schwarz, 2005).

1.4.1.2 UV-induced immunosuppression

There is increasing evidence showing that UVB therapy induces both local and systemic immunosuppressive effects which may contribute to the clinical efficacy of NB-UVB phototherapy (Schwarz, 2008). UVB has been suggested to modulate LCs to induced local immunosuppressive effects. Low dose UVB has been shown to promote the emigration of LCs to cutaneous lymph nodes while high dose UVB induced apoptosis of LC, both of which lead to decreased number of LCs in the epidermis. Besides, antigen presenting ability of LCs is also inhibited by UVR, which has been proven both *in vitro* and *in vivo*. UVB induced down-regulation of CD80/CD86 expression on LCs and blood derived DCs which contribute to the inhibition of their antigen presenting ability (Schwarz, 2005a). Keratinocytes with

UVB-damaged DNA release IL-10, which affects antigen presentation by LCs and contributes to the systemic immunosuppression of UVB (Nishigori et al., 1996). Neutralizing IL-10 reverses UVR induced systemic immunosuppression of induction of delayed type hypersensitivity.

1.4.1.3 UV-induced Tregs

One of the major hallmarks of UVB-induced immunosuppression is that it acts in an antigen-specific fashion, thus differing from the more general immunosuppression induced by immunosuppressive drugs. UVB-induced, antigen specific, Tregs have been characterized in several murine model and Tregs with unique phenotypes are involved. The best characterized UV-induced Tregs are those in the experimental murine model of contact hypersensitivity. They express CD4, CD25, CTLA-4, dectin-2, glucocorticoid-induced TNF receptor family–related protein (GITR), neuropilin, and CD62L but not the ligands for the skin-homing receptors E- and P-selectin. A small subtype of UV-induced Tregs expresses the transcription factor FoxP3 (Schwarz, 2005b). Tregs have to be activated in an antigen-specific manner to exert their suppressive function. *In vitro* activation with the specific antigen induced the release of IL-10 by UV-induced Tregs. One of the mechanisms by which UV irradiation induces Tregs is through LCs. UVB damaged but viable LCs is required in regional lymph nodes for the generation of Tregs (Schwarz et al., 2010). Besides this mechanism, receptor activator of NF- κB (RANK) and its ligand RANKL are also involved in the induction of UV-induced Tregs. Enhanced number of CD4+CD25+ cells are observed in mice that overexpress RANKL in basal keratinocytes. RANKL expression on keratinocytes induced by UVB irradiation activates LCs leading to the generation of Tregs (Loser et al., 2006). Murine

antimicrobial peptide β-defensin-14 induced by UV irradiation converts CD4+CD25- effector T cells into functional Foxp3+Tregs leading to inhibition of sensitization upon adoptive transfer into naïve mice in contact hypersensitivity model (Navid et al., 2012). Taken together, these results suggest that multiple pathways are involved in UV-induced Tregs generation.

1.4.1.4 UV-induced anti-inflammatory molecules

It is well known that UV radiation induces the production of vitamin D, which is considered as an important immune regulatory molecule. Topical vitamin D analogs have been successfully used in treating psoriasis. Therefore, it is suggested that UV-induced vitamin D is responsible for the clinical beneficial effect of UVB phototherapy. However, UV radiation could suppress immune responses independently of vitamin D (Schwarz et al., 2012), as further discussed below.

1.4.1.4.1 UV-induced vitamin D

UVB photolyses 7- dehydrocholesterol to cholecalciferol in the skin, which is then hydroxylated in the liver and kidney to form the most active form of vitamin D $1,25(OH)_2D_3$ (Lehmann et al., 2001)

$1,25(OH)_2D_3$ (vitD) is an important mediator induced by UVB therapy with immune regulatory functions which play an important role in preventing progression of AD. VitD impairs the differentiation of monocytes into DCs and decreases the expression of co-stimulatory molecules and secretion of cytokines from cultured monocytes and DCs (Baeke et al., 2010). VitD-modulated skin-derived LC and dermal DC induce Foxp3-expressing and

IL-10-secreting Tregs, respectively (van der Aar et al., 2011). In addition, vitD has direct effects on CD4+ T cells by inducing the development of Foxp3+Treg and enhancing the secretion of IL-10 (Etten and Mathieu 2005). VitD also has a direct effect on B cells by reducing their differentiation into memory and plasma B cell subtypes and their capacity to produce antibody (Etten and Mathieu, 2005).

1.4.1.4.2 UV-induced nitric oxide (NO) generation

The nitrate–nitrite–NO pathway in normal physiology and in pathological hypoxia complements the classical L-arginine-NOS pathway, which is largely dependent on oxygen to ensure NO production in situations for which the oxygen-dependent NOS enzyme activities are compromised.

1.4.1.4.2.1 Sources for NOS-independent NO generation

Nitrate, nitrite and RXNO (the sum of S-nitrosothiols (RSNOs), N-nitrosamines (RNNOs), iron-nitrosyl species) form the major nitro-species present in skin and in circulation and serve as a stable endocrine carrier of NO (Kelm, 2005). Endogenous NO, generated by NOS enzymes, is oxidized in the blood and tissues to form nitrite, a process catalysed by the multi-copper oxidase and NO oxidase ceruloplasmin in plasma (Shiva et al., 2006). It has been shown that nitrite has short half-life in whole blood (±110 seconds) and rapidly oxidizes to nitrate in the range of minutes. Nitrate in human plasma compared to nitrite has a longer half-life of approximately 8 hours, and this forms the largest store of nitro-species (Moshage et al., 1995). Dietary intake is one of the major sources of nitrate in the body (Lundberg and Weitzberg 2005). Besides this, endogenous NO can also form nitrate by reacting with oxyhaemoglobin in red blood cells. RXNO are derivatives of nitric oxide, while RSNOs are

formed by S-nitrosation of thiols and RNNOs are formed due to the reaction of nitrite with amino groups of food constituents in the acidic environment of stomach.

1.4.1.4.2.2 Mechanisms for NOS-independent NO generation

Commensal bacteria convert nitrate to nitrite via one–electron reduction in humans since mammals do not synthesize nitrate reductase. Commensal bacteria involved in this process include:- bacteria in the oral cavity among which *Veillonella* species (*V.atypica* and *V.dispar*) are the most prevalent nitrate reducers (Doel et al., 2005); bacteria on body surfaces such as *S.aureus, S.epidermidis,* and Nitrosomonas species (Weller et al., 1996); and, bacteria in the gastrointestinal tract such as Lactobacillus species (*L.rhamnosus, L.acidophilus*) and Bifidobacterium species (*B.longum infantis*) (Tiso and Schechter, 2015).

In the acidic condition of the stomach, nitrite was found to rapidly generate nitrous acid (HNO_2), which further decomposes to form NO (Benjamin et al., 1994). Reducing compounds such as vitamin C and polyphenols, both of which are abundant in the diet are suggested to enhance the process of reduction of nitrite to NO.

Nitrate and nitrite recycle in blood and tissue and act as NO-storage to generate NO and other bioactive nitrogen oxides when oxygen tensions falls. It was reported by Doyle et al in 1981 that nitrite reacts with deoxyhemoglobin to generate NO in regions of low oxygen tension where deoxyhemoglobin predominates (Doyle et al., 1981). The expression of xanthine oxidoreductase, the structure of which is related to bacterial nitrate and nitrite reductases, is greatly enhanced at low oxygen tensions and acidic conditions. It has been shown that xanthine oxidoreductase catalyses the reduction of nitrite or nitrate to NO (Lundberg and Weitzberg, 2005).

Adnana N. Paunel *et al* showed that upon exposure to UVA, nitrite and RSNO contained in human skin undergo photolysis to generate NO, the concentration of which is comparable or even higher than that produced by cytokine-activated human keratinocyte cultures with maximal iNOS activity *in vitro* (Paunel et al., 2005). This process is enhanced by thiols *in vivo* as shown by Dejam and colleagues who reported that upon UV irradiation, thiols (which are a major component of cysteine and glutathione in keratinocytes) enhance the non-enzymatic conversion of nitrate to nitric oxide and nitric oxide-adducts (Dejam et al., 2003).

1.4.1.4.3 UV induced *cis*-urocanic acid (*cis*-UCA) generation

Urocanic acid (UCA), located in the stratum corneum, is a major epidermal chromophore and suggested to play a role in UV-induced immunosuppression. UCA is a histidine-derived molecule produced by histidase, which is converted from *trans*-UCA to *cis*-UCA after UV radiation. Urocanase, which catabolizes UCA to imidazolonepropionic acid and subsequently to glutamic acid, exists in liver but is absent in skin leading to accumulation of trans-UCA which takes up to 0.5% of the dry weight of the epidermis. It is suggested that the epidermal concentration of UCA varies from 4 to 34nM/cm^2, and this does not correlate with age, gender, pigmentation, skin phototype, or minimal erythema dose (Gibbs et al., 2008). Recent studies suggest that the level of urocanic acid in stratum corneum is associated with FLG genotype and severity of AD (Kezic et al., 2011).

Photoconversion of trans to cis-UCA is mainly induced by wavelengths between 310 and 340 nm (Berneburg et al., 2005). Photoprotection provided by the conversion of trans-*cis* UCA is equal to a sunscreen with a sun protection factor (SPF) of 1.5. The major source of histidine in the skin is filaggrin (Denecker et al., 2007). Therefore, FLG mutation results in a lower level of endogenous UCA, which acts as a constitutive photo protectant that could reduce UV

radiation induced deleterious effect by 33% (Gibbs and Norval, 2011).

Taken together, these evidence suggest that NB-UVB releases other potential anti-inflammatory molecules than vitD. I further investigated the function of NO and *cis*-UCA on human immune cells and their contribution to the clinical beneficial effect of NB-UVB phototherapy.

Hypothesis

NO and *cis*-UCA contribute to the clinical efficacy of NB-UVB phototherapy in AD through their effects on the function of regulatory T cells either directly, or indirectly through its action on antigen presenting cells.

Aims

- Study the effects of NO on the phenotype and function of human CD4+ T cells and mo-DCs from healthy volunteers.

- Study the effects of *cis*-UCA on the phenotype and function of human CD4+ T cells and mo-DCs from healthy volunteers.

- Study whether the clinical efficacy of NB-UVB phototherapy in AD is linked to changes in the phenotype of T cells and Tregs

- Study the correlation between vitamin D/Nitrate/FLG genotype, circulating Tregs and clinical efficacy of NB-UVB phototherapy in AD.

Chapter 2 Materials and Methods

2.1 Reagents

2.1.1 Buffers

2.1.1.1 PRMI tissue culture medium

Complete RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (all Gibco, Life Technologies, Paisley, UK).

2.1.1.2 DC culture medium

RPMI 1640 buffered with 20mM HEPES (Sigma, Poole, UK), 5% AB serum (Sigma), 2 mM L-glutamine (Gibco), 50ng/ml GM-CSF, 15ng/ml IL-4 (both Miltenyi, Surrey, UK)

2.1.1.3 FACS Buffer

PBS without Ca^{2+} and Mg^{2+} supplemented with 2% heat-inactivated FBS (both Gibco) and 0.05% sodium azide (Sigma)

2.1.1.4 MACS buffer

PBS without Ca^{2+} and Mg^{2+} supplemented with 2% heat-inactivated FBS and 1mM EDTA (All Gibco)

2.1.1.5 Monocyte wash buffer

PBS without Ca^{2+} and Mg^{2+} supplemented with 0.5% human AB serum and 2nM EDTA

2.1.1.6 Protein lysis buffer

Radio Immunoprecipitation Assay (RIPA) buffer containing 25 mM Tris·HCl pH 7.6, 150mM

NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS supplemented with protease inhibitor cocktail (Thermo Scientific, Loughborough, UK) and 0.5% Na_3PO_4.

2.1.1.7 Western blot sample buffer

3X Sample buffer contains 0.19 M Tris pH 6.8, 6% SDS, 30% glycerol, 0.3M DTT, 0.02% bromophenol blue and dilutes with samples to 1X when needed.

2.1.1.8 Western blot running buffer

10X Running buffer contains 1.92M Glycin, 0.25M Tris base, 10% (v/v) SDS.

2.1.1.9 Western blot transfer buffer

Transfer buffer contains 25mM Tris base, 192mM glycine and 20% (v/v) methanol.

2.1.1.10 Western blot washing buffer

10X TBST Wash Buffer stock was prepared (0.1M Tris HCl, 1.5M NaCl, 0.5% Tween-20). It was diluted to 1X TBST with Milli Q water and used as western blot washing buffer.

2.1.1.11 Western blot blocking buffer

5% w/v non-fat dry milk dissolved in 1X TBST.

2.1.1.12 Western blot stripping buffer

Stripping buffer contains 2%SDS, 62.5 µM Tris HCL pH6.8 and 0.8% ß-mercaptoethanol

2.1.1.13 Antibodies

The following antibodies were used for FACS staining (all from Biolegend, London, UK

except where stated). For clones see Antibody Table 2.1.

Surface staining: Viability dye eFluor® 780 (ebioscience), anti-CD3 AF700, anti-CD4 FITC (BD Pharmingen), anti-CD4 PerCP, anti-CD25 AF488, anti-CD25 APC (BD Pharmingen), anti-CLA Pacific Blue, anti-CD127 PE (ebioscience), anti-CD8 PE, anti-CD69 PE, anti-CD46 FITC, anti-PD-1 PerCP, anti-PD-L1 PE-Cy7, anti-CD14 Pacific Blue, anti-HLA-DR PE-Cy5, anti-CD11c APC, anti-CD86 AF488, anti-CD86 APC, anti-CD80 AF700, anti-CD83 APC, anti-CD40 PE-Cy7,

Intracellular staining: anti-Foxp3 PE (BD Pharmingen), anti-Foxp3 AF647

Table 2.1 Flow cytometry antibody clones and dilutions

Antibody	Conjugate	Clone	Dilution
CD3	AF700	UCHT1	1:100
CD4	FITC/PerCP	RPA-T4	1:100
CD25	AF488	BC96	1:200
	APC	M-A251	1:100
CLA	Pacific Blue	HECA-452	1:200
CD127	PE	eBioRDR5	1:25
CD8	PE	SK1	1:100
CD69	PE	FN50	1:100
CD46	FITC	MEM-258	1:200
PD-1	PerCP	EH12.2H7	1:100
PD-L1	PE-Cy7	29E.2A3	1:100
CD14	Pacific blue	HCD14	1:100
HLA-DR	PE-Cy5	L243	1:100
CD11c	APC	Bu15	1:100
CD86	AF488/APC	IT2.2	1:100

CD80	AF700	L307.4	1:100
CD83	AF488	HB15e	1:100
Foxp3	PE	259D/C7	1:100
	AF647	206D	1:50

2.1.2 Chemical preparation

All these chemicals were purchased from Sigma except where stated and were diluted to the required concentration using cell culture medium in each experiment.

2.1.2.1 *Cis*-UCA preparation

Cis-UCA stock was prepared by reconstitution at the concentration of 10 μg/μl using complete RPMI 1640.

2.1.2.2 Trans-UCA preparation

Trans-UCA stock was prepared by reconstitution at the concentration of 1 μg/μl using complete RPMI 1640.

2.1.2.3 Vitamin D (vitD) preparation

vitD stock was prepared by reconstitution at the concentration of 10^{-4}M using 95% sterilized ethanol.

2.1.2.4 Diethylenetriamine/nitric oxide adduct (DETA-NO) preparation

NO-donating compound DETA-NO was prepared by reconstitution at the concentration of 10mM in complete RPMI.

2.1.2.5 NOC-18 preparation

Stable NO-donating compound 3,3-Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC-18) was prepared by reconstitution at the concentration of 10mM in complete RPMI.

2.1.2.6 Ketanserin (+)-tartrate salt preparation

Ketanserin (+)-tartrate salt (Ke) was prepared by reconstitution at the concentration of 10mM in DMSO.

2.1.2.7 α-Methylserotonin maleate salt preparation

α-Methylserotonin maleate salt (α-Me) was prepared by reconstituted at the concentration of 10mM in complete RPMI.

2.1.2.8 LPS preparation

LPS stock was prepared by reconstitution at the concentration of 10 mg/ml in PBS.

2.2 Cell isolation and purifications

2.2.1 Plasma isolation

Periphery blood was collected from informed consented volunteers using 3.8% sodium citrate as anticoagulant. Plasma was isolated from undiluted blood by centrifuging at 445g for 10 min at 4 ℃. Following centrifugation, the resulting supernatant was aliquoted into 0.5 ml aliquots and stored at -80 ℃.

2.2.2 PBMC isolation from peripheral blood

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque PLUS (GE Healthcare Life Sciences, Buckinghamshire, UK) density gradient centrifugation.

Approximately 40ml blood was collected into a 50 ml falcon tube with 4ml of 3.8% sodium citrate. Blood was diluted 1:1 with PBS in a 75 cm^2 culture flask. 27 ml of diluted blood was layered onto 13ml Ficoll in 50ml falcon tube and centrifuged at 650g for 25 min without break. The PBMCs interphase was aspirated into a fresh 50 ml falcon tube and washed with PBMC wash buffer once by centrifuging at 300g for 10min. Then the cell pellet was washed another 3 times at 200g for 10 min.

2.2.3 CD14+ monocytes purification

CD14+ monocytes were isolated by positive selection with Human CD14 selection kit from STEMCELL (Manchester, UK) according to the manufacturer's instructions. PBMC were resuspended at a concentration of $1*10^8$ cells/mL in MACS buffer and incubated with EasySep positive selection antibody cocktail at 100 μL/mL cells for 15 minutes at room temperature. CD14+ monocytes were targeted with tetrameric antibody complexes recognizing CD14 and magnetic particles. Cells were then incubated with EasySep Magnetic Nanoparticles at 50 μL/mL for 10 minutes before brought up to 2.5mL by adding MACS buffer and incubated in Purple EasySep Magnet for 5 minutes. The unwanted CD14- cell populated were poured off by inverting the magnet and tube and the magnetically labelled cells were remained inside the tube holding by the magnetic field of the Purple EasySep Magnet. An aliquot of the cells were stained with a Pacific Blue-conjugated CD14 antibody to assess cell purity by FACS analysis. Purity of the monocytes was consistently >95%.

2.2.4 CD4+ T cell purifications

CD4+ T cells were isolated by negative selection with Human CD4 T cell enrichment kit from STEMCELL (Manchester, UK) according to manufacturer's instructions. CD14- cells after monocytes isolation were re-suspended at $5*10^7$ cells/mL in MACS buffer and

incubated with EasySep Human CD4+T Cell Enrichment Cocktail at 50 µL/mL cell for 10 minutes at room temperature. Unwanted cells were targeted for removal with tetrameric antibody complexes recognizing CD8, CD14 ect. Cells were then incubated with EasySep™ D Magnetic Particles at 100 µL/mL cells for 5 minutes before brought up to 2.5mL by adding MACS buffer and incubated in Purple EasySep Magnet for 5 minutes. The un-touched CD4+ T cells were poured off by inverting the magnet. An aliquot of the cells were stained with a FITC-conjugated CD4 antibody to assess cell purity by FACS analysis. Purity of the CD4+ T cells was consistently >95%.

2.2.5 Positive selection of CD4+CD25+ T cells

CD4+CD25+ T cells were isolated by positive selection with EasySep Human CD4+CD25high T Cell Isolation Kit from STEMCELL (Manchester, UK) according to manufacturer's instructions. The selected CD4+ T cells (see section 2.2.4) were incubated with Easysep blocking buffer at 50 µl/ml for 15 minutes at room temperature. After washing twice with MACS buffer, cells were incubated with EasySep CD25 Positive Selection Cocktail at 25 µl/ml for 15 minutes and then incubated with EasySep Magnetic Nanoparticles at 12.5 µl/ml for 10 minutes. Magnetically labelled CD4+CD25+ T cells were then isolated using Purple EasySep Magnet.

2.2.6 Negative selection of CD4+CD25- T cells

CD4+CD25- T cells were isolated by negative selection with EasySep Human CD4+CD25high T Cell Isolation Kit from STEMCELL (Manchester, UK) according to manufacturer's instructions. The selected CD4+ T cells (see section 2.2.4) were incubated with Easysep blocking buffer at 50 µl/ml for 15 minutes at room temperature. After washing twice with MACS buffer, cells were depleted of CD25 expressing cells by incubation with

EasySep CD25 Positive Selection Cocktail at 100 µl/ml for 15 minutes to and then incubated with EasySep Magnetic Nanoparticles at 100 µl/ml for 15 minutes. Magnetically labelled CD4+CD25+ T cells were isolated using Purple EasySep Magnet. The CD4+CD25- cells were poured off by inverting the magnet and tube and the magnetically labelled unwanted CD25+ cells remained inside the tube held by the magnetic field of the Purple EasySep Magnet.

2.3 Methods

2.3.1 *In vitro* cell culture

2.3.1.1 Mo-DCs differentiation and maturation

CD14+ monocytes were seeded in 24-well NUNC plates (Fisher Scientific, Loughborough, UK) at 2* 10^6 cells per ml per cm2 in DC culture medium with or without different treatment. Cells were cultured at 37 ℃ in a humidified atmosphere of 5% CO2. One third medium with or without treatment was added on day 3 and day 5. On day 7, cell suspension was collected by centrifuging at 300g for 10 min without brake at room temperature. A fraction of the cells were removed for cell count and immature DC phenotype analysis. The spare cells were re-plated at 0.5*10^6 cells/ml in DC culture medium with addition of 20 µg/ml Polyinosinic-polycytidylic (Poly I/C) for 48h or addition of 10 ng/ml LPS for 24 hours. Cells were collected for mature DC phenotype analysis and supernatants were used for ELISA.

2.3.1.2 CD4+ T cell activation

48-well plates were pre-coated with anti-human CD3 (OKT3, ebioscience) and anti-human CD28 (CD28.2, ebioscience) at the concentration of 1 to 5 µg/ml for 2 hours at 37°C. CD4+ T cells were then seeded at 0.5*10^6 cell/ml in complete RPMI1640 with or without different treatments. Cells were cultured at 37 ℃ in a humidified atmosphere of 5% CO2. Cells were

collected after 5 days for phenotype analysis and supernatants were used for ELISA.

2.3.2 FACS analysis

2.3.2.1 Surface staining

For surface staining, cells were centrifuged at 445g for 5 minutes and then resuspended in FACS buffer in 96-well round bottom plate. The cell pellet was washed twice with PBS before incubating with 50 µl of diluted Viability dye eFluor® 780 for 20 minutes at 4℃ protected from light. After 2 washes with FACS washing buffer, 50 µl FACS wash containing 2% mouse serum was added and the tube was incubated on ice for 10 mins to block Fc receptor binding. Cells were then incubated with 50 µl surface staining antibody cocktail and incubated at 4℃ protected from light for 20mins. Cells were washed with FACS buffer once and re-suspended in 300 µl FACS washing buffer. Cells were ready to be analysed on FACS Calibur (Becton Dickinson) or Fortessa (BD) flow cytometers.

2.3.2.2 Intracellular staining

For intracellular staining, Foxp3 was stained using Foxp3 staining buffer set from BD following the manufacturer's instructions. Cells were surface stained as described above then incubated in fixation buffer for 10 minutes. Cells were washed with FACS buffer then incubated with the permeabilisation buffer at room temperature for 30 minutes. Cells were washed with FACS buffer twice before stained with anti-Foxp3 PE/AF488 (BD/Biolegend) for 30 minutes at room temperature. Cells were washed in FACS buffer then re-suspended in 300µl FACS buffer for collection at a rate of ~500 events/second.

2.3.2.3 Whole blood staining

After removing the serum, blood cell pellets were diluted with a same volume of ice-cold

FACS buffer. 50μL of cell suspension were added to a round bottom 96-well plate and stained with surface staining or intracellular staining as described in 2.3.2.1 and 2.3.2.2. Cells were then incubated with 200 μL of FACS-Lysing solution for 8 minutes at room temperature protected from light. The lysing process was repeated till wells appear free of haemoglobin. Cells were re-suspended in 200 μL FACS buffer and analysed using the Fortessa (BD) flow cytometer.

2.3.2.4 Proliferation assay

CD4+ T cells were stained with the proliferation dye ef670 (ebioscience) following the manufacturer's instructions. In brief, cells were incubated with diluted proliferation dye ef670 for 10 minutes at 37℃ in the dark in PBS. Cells were then incubated on ice for 5 minutes with complete RPMI medium to quench the reaction before being washed three times with complete RPMI 1640 tissue culture medium. Cells were then re-suspended complete in RPMI 1640 culture medium at desired concentration and cultured for 4 days .

2.3.2.5 Suppression assay

Regulatory T cell suppressive function assays were set up in 96-well U-bottom plates. In brief, total CD4+ T cells stained with the proliferation dye ef670 as described in 2.3.2.4 ($5*10^4$ cells/well used as responders) were co-cultured with different numbers of freshly purified CD4+CD25+ T cells as suppressor cells in the presence of pre-coated anti-CD3/CD28 (5μg/ml) in 200 μl of RPMI1640 tissue culture medium. The proliferation of the responder cells were analyzed using using the Fortessa (BD) flow cytometer.

2.3.2.6 Transwell assay

Transwell assays were set up in 24-well plates. In brief, total CD4+ T cells stained with the

proliferation dye ef670 as described in 2.5.1.4 (10x 10^5 cells/well used as responders) were cultured in the presence of pre-coated anti-CD3/CD28 (5μg/ml) in 800 μl of RPMI1640 tissue culture medium. Different numbers of NO-treated CD4+ T cells as suppressor cells were seeded in the transwell insert which separates suppressor cells from responder cells. The proliferation of responder cells were analyzed using using the Fortessa (BD) flow cytometer.

2.3.3 Cytokine array

The cytokine profile secreted by CD4+ T cells was analysed using the Proteome Profiler Human Cytokine Array Panel A kit (R&D Systems) according to the manufacturer's protocol. Cell culture supernatants of activated CD4+ T cells having the same treatment (*cis*-UCA or vitD or NO) from 9 healthy donors were combined together and used as samples to avoid variability of individual donors. Pre-coated membrane was blocked with 2ml Array buffer 4 for an hour. 15 μL of reconstituted human cytokine array panel A detection antibody cocktail was mixed with 1.5ml samples and incubated for an hour before the antibody/sample mixtures being incubated with pre-coated membrane overnight at 4 degree. After being washed with wash buffer for 3 times, pre-coated membrane was incubated with 2ml diluted Streptavidin-HRP for 30min. After being washed 3 times, pre-coated membrane was incubated with 1 mL of the prepared Chemi Reagent Mix and exposed to X-ray film for 1-10 minutes.

2.3.4 Enzyme linked immunosorbent assay (ELISA)

The ELISA assay was carried out as per manufacturer's instructions (R&D Systems). Briefly, flat-bottomed 96-well plates were coated overnight at room temperature with 100 μL of coating antibody. Plates were washed 3 times with wash/dilution buffer (0.1% Tween 20 in PBS pH 7.2) between each treatment. 200 μL/well of blocking buffer (1% ovalbumin in PBS)

was added to the plates and incubated for 1 h before being treated with 100 µL of either sample or standard for 2 h at room temperature while shaking. Wells were then treated with 100 µL of biotinylated detection antibody per well and incubated for 2 h at room temperature followed by 100 µL of streptavidin–horseradish peroxidase, diluted 1 in 200 diluent buffer, for 20 min at room temperature while shaking. After washing 2 times, 100 µL of substrate solution was added to each well and allowed to incubate for 30 min. The reaction was stopped by the addition of 50 µL of 2N sulfuric acid. The absorbance was quantified at an emission of 450 nm with reference wavelength at 590 nm (Dynex MRX plate reader).

2.3.5 RNA extraction and Real-time PCR

2.3.5.1 RNA extraction and quantification

After 3 days culture, cell pellets were collected by centrifugation at 445g for 5 minutes. RNA was isolated using the RNeasy Micro Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. RNA concentration was assessed by spectrophotometry using a NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK).

2.3.5.2 Complementary DNA (cDNA) Synthesis

Depending on the number of CD4+ T cells obtained, the RNA concentration was usually between 10-20 ng/ µl. 12 µl RNA was used for the reverse transcriptase reaction to synthesize cDNA using Qiagen Quanti Tect Rev Transcription Kit. Briefly, 12 µl RNA was incubated with 2 µl gDNA wipe out buffer for 5 minutes at 42°C. The DNase-treated RNA was subsequently reverse transcribed in 20 µl reactions according to the manufacturer's instructions. cDNA synthesis was allowed to proceed for 25 minutes at 42 ℃ before inactivation at 95 ℃ for 3 minutes. cDNA was stored at –20 ℃ till further use.

2.3.5.3 Real-time quantitative polymerase chain reaction (PCR)

The expression of VDR was measured by real-time qPCR using relative quantification normalized to 18S rRNA. PCR was performed in 24-well PCR plates in a 20 µl reaction volume (2 µl of cDNA, 1µl 18s rRNA primers and probe, 1µl VDR primers and probe, 10 µl of Taqman PCR Master Mix and 6 µl H2O) and PCR (40 cycles) was run in the condition as follows: The PCR cycle parameters were 95℃ for 10 minutes, 40 cycles of 95℃ for 2 seconds, and 60℃ for 20 second. The PCR data were acquired and quantified by relative quantification (RQ) analysis $RQ=2^{-\Delta\Delta CT}$. RQ value showed the fold change in gene expression compared with control group that was normalized to 1.

2.3.6 Western blot

2.3.6.1 Protein extraction

CD4+ T cells were centrifuged at 445g and washed with ice cold PBS twice. They were re-suspended in 30µl of protein lysis buffer and incubated on ice for 15 min before being centrifuged at 445g at 4 ℃ for 15 min. The supernatant was removed, aliquoted and stored at -20 ℃ until being used in western blot experiments.

2.3.6.2 Bicinchoninic acid (BCA) protein assay

Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Loughborough, UK) according to the manufacturer's manual. 10 µl of a protein sample was used in the assay. Samples were stored at -80℃ until used in western blot experiments.

2.3.6.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Proteins were denatured by heating to 95℃ for 3 minutes in western blot sample buffer. 8-12%

running gel and 5% stacking gel were prepared for separation (See Table 2 for the composition for running and stacking gel). Once set, equal amounts of proteins were loaded into wells and the Prism Ultra Protein Ladder (10-180 kDa) was used as molecular weight markers. The gel was electrophoresed at 150 V until the dye front was close to the bottom. After running the SDS-PAGE, the gel was transferred to a polyvinyl difluoride (PVDF) membrane in western blot transfer buffer for 1 h at 100V. Membrane was blocked in western blot blocking buffer for 1 h with shaking at room temperature before incubating with the primary antibody diluted in blocking buffer overnight at 4 degree. After three washes of 10 minutes with TBST, membrane was incubated with the secondary antibody for 45-60 minutes. The membrane was washed 3 times with washing buffer before being developed by enhanced chemiluminescence (ECL).

Table 2.2 Composition for running and stacking gels

Running gel	
Acrylamide 30%	4ml
Tris 1.5M pH 8.8	3ml
H20	4.8ml
SDS 10%	120µl
APS 10%	54µl
Temed	9µl

Stacking gel	
Acrylamide 30%	1ml
Tris 1.5M pH 6.8	1.25ml
H20	2.75ml
SDS 10%	50µl
APS 10%	25µl
Temed	5µl

2.3.6.4 Stripping and re-probing of PDVF membranes

To allow a western blot membrane to be re-probed, it was stripped using striping buffer. In brief, membrane was washed twice with TBST before being incubated with stripping buffer for 15 minutes at 56 ℃. It was then washed twice with TBST with gentle agitation and blocked with blocking buffer for 1 h. The membrane was then incubated with another antibody as previously described in section 2.5.5.2.

2.3.6.5 Densitometry analysis

Blots were scanned and densitometric analysis was carried out using ImageJ software. Full length of protein bands were selected manually and the net pixel intensity of the selected bands were measured.

2.3.7 Nitrate and nitrite analysis

Concentration of nitrate and nitrite in plasma was analyzed using Nitric Oxide Assay Kit (Thermo Scientific, Loughborough, UK) according to the manufacturer's instructions. In brief, plasma was diluted using Reagent Diluent provided in the kit and ultra-filtrated through a 10,000 molecular weight cut off filter. For nitrite analysis, 50 µl of flitted plasma was incubated with 50 µl Griess Reagent I and Griess Reagent II for 10 min at room temperature and optical density of each well at 540 was detected using Dynex MRX plate reader. Nitrate in the plasma was converted to nitrite by incubating with NADH and nitrate reductase enzyme at 37 ℃ for 30min before analysis.

2.3.8 VitD analysis

Concentration of 25 (OH) vitamin D in plasma was analyzed using 25(OH) Vitamin D ELISA kit (Sigma) according to the manufacturer's instructions.

2.3.9 FLG genotyping

All patients were screened for FLG mutations and the genotyping was performed by the Wellcome Trust Clinical Research Facility. The two FLG mutations that were most common in Caucasian populations (2282del4, R501X) were tested. R501X mutation arises from a 1501C-to-T transition near the start of repeat 1 in exon 3 of the FLG gene. The 2282del4 mutation leads to a premature termination codon 107 bp downstream and, like R501X, stops protein translation within the first filaggrin repeat.

Genomic DNA was isolated from whole blood. Genotyping was performed in 384 well-plates, using the TaqMan polymerase chain reaction-based method. Allelic discrimination using this chemistry is based on the design of two TaqMan probes, specific for the wild type allele and the mutant allele. Each of the two probes is labelled with a different fluorescent tag (FAM and VIC), and each is designed with the gene mutation affecting the middle part of the probe sequence. The binding efficiency of the wild type TaqMan probe to the mutant allele and vice versa is low due to the mismatch within the TaqMan probe and the target sequence; therefore, mismatched binding is highly reduced.

The final volume PCR reaction was 10 µl using 20 ng of genomic DNA, 5 µl of Universal PCR Taqman Master Mix and 0.06µl of Primer, 0.02ul of Probe and 4.92µl of deionised water or 0.1µl of Primer, 0.01µl of Inner Primer, 0.02µl of Probe and 4.87uµl of deionised water.
The cycling parameters are as follows: 95 ° for 10 minutes, followed by forty cycles of denaturation at 95 ° for 15 seconds and annealing/extension at 60 ° for 1 minute. PCR plates were then read on Applied Biosystems by Life Technologies QuantStudio 12K Flex Real Time PCR System (#285880765) instrument with QuantStudio 12K Flex Software or Taqman Genotyper Software v1.3.

2.4 Statistical analysis

All data are expressed as mean±SEM. Data were analyzed, where appropriate, using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P value of less than 0.05 was considered significant.

Chapter 3 Investigating the effect of nitric oxide on human peripheral CD4+ T cell differentiation and function

3.1 Introduction

UV radiation, especially the middle wavelength UVB (290nm-320nm), induces both local and systemic immunosuppression. It has been shown in a murine contact hypersensitivity (CHS) model that application of haptens on UV-exposed skin failed to induce sensitization, and that adoptively transferred splenocytes obtained from these animals resulted in hapten-specific unresponsiveness in recipient animals. Depletion of T cells from the transferred cells resulted in loss of suppressive activity which indicated that T cells with regulatory function were important in UV-induced immunosuppression (Schwarz, 2008).

The phenotype of UV-induced murine antigen specific regulatory T cells has been characterized in the CHS model. It was demonstrated that UV-induced Treg express CD4, CD25, CTLA-4, dectin-2, glucocorticoid-induced TNF receptor family–related protein (GITR), neuropilin, and CD62L but not the ligands for the skin-homing receptors E- and P-selectin. A small subtype of murine UV-induced Treg expressed the transcription factor FoxP3 (Schwarz, 2005). *In vitro* activation with the specific antigen induced the release of IL-10 by UV-induced Treg, which was critically involved with the suppressive activity.

NO is reported to play a key role in cutaneous physiology. Topical application of the NO inhibitor NG-monomethyl-L-arginine acetate (L-NMMA) resulted in a reduced level of UV induced immunosuppression in humans, suggesting that UV-induced NO played a role in UV radiation-induced immunosuppression (Kuchel et al., 2003). Skin contains large stores of nitrogen species, which contribute to increase the circulating NO metabolite pool. Studies

have shown that nitrite in skin was photolysed on irradiation with UV to release NO (Mowbray et al., 2009). Nitrate photolysis to release NO was greatly enhanced by the thiols-containing amino-acid cysteine, which was abundant in skin (Dejam et al., 2003). These data suggest that translocation of NO from skin to the circulation on exposure to UV radiation may contribute to the control of systemic inflammatory responses.

Most of the physiological activities of NO are mediated through soluble guanylyl cyclase (sGC) which is the key enzyme in NO signalling pathway (Calabrese, V., C. Mancuso, et al, 2007). NO activates sGC and in turn initiates the production of the messenger cGMP, which inhibits leukocyte recruitment, excessive cell proliferation and inflammation through a number of downstream mechanisms.

The role of NO in regulatory T cells (Tregs) differentiation remains controversial. Murine CD4+ T cells have been reported to express iNOS upon activation, which suppressed the differentiation of these cells into Foxp3+Treg induced by TGFβ, although the suppressive function of induced Foxp3+Treg remained unaffected (Jayaraman et al., 2014). Similarly, it has been shown that NO inhibited Foxp3+Treg induction driven by TGFβ and promoted naive CD4+ T cell differentiation to Th1 in murine model (Lee et al., 2011). Furthermore, in a murine model of multiple sclerosis, NO produced by splenocytes from myelin basic protein-immunized mouse decreased CD4+CD25+ and CD25+Foxp3+ population, which was further confirmed by using splenocytes from iNOS knock out mice. The effect of NO on Foxp3 expression was mediated by soluble guanylyl cyclase-mediated production of cGMP (Brahmachari and Pahan, 2010).

Whilst these data demonstrate the importance of NO in suppressing Foxp3+Treg

differentiation, there are also reports suggesting that NO is involved in Treg induction in mice. Systemic NOS inhibition using L-NG-nitro arginine methyl ester (L-NAME) decreased Treg in circulation (Brinson et al., 2013). Exogenous NO could induce the generation of a type of regulatory T cell (NO-Treg) from murine naive CD4+ T cells that did not express Foxp3 but secreted IL-10 and required cell-cell contact for its suppressive effect (Niedbala et al., 2007). Stimulation of CD4+ T cells by allogeneic bone marrow-derived dendritic cells (DC) in the presence of IFN-γ resulted in Treg induction. This effect was abolished by NOS inhibition and could be replicated by provision of an exogenous NO donor, which clearly indicated the involvement of NO in this process (Feng et al., 2008).

These results highlight the complex role of NO in murine Treg induction, but no conclusive data exist so far on the effect of NO on human Treg differentiation. A previous study reported that similarly to murine CD4+ T cells, iNOS expression in human CD4+ T cells also inhibited Foxp3 expression and Treg induction driven by TGFβ (Jayaraman et al., 2014). In contrast, Niedbala et al reported that NO-Treg were induced by NO from human peripheral CD4+ T cells (Niedbala et al., 2007).

As understanding the mechanisms governing Foxp3+ Treg induction is important for AD treatments, this chapter describes experiments designed to determine the role of NO in the induction of Foxp3+Treg from human peripheral CD4+ T cells.

Chapter hypothesis and research questions

NO contributes to the clinical efficacy of NB-UVB phototherapy in AD through their effects on the function of regulatory T cells either directly or indirectly through its action on antigen presenting cells.

- Does NO induce functional regulatory T cells from human peripheral CD4+ T cells?
- Does NO modulate CD4+ T cell function directly or indirectly through antigen presenting cells?

3.2 Results

3.2.1. NO modulates human peripheral CD4+ T cell phenotype.

NOC-18 is a NO donor which releases NO at a slow rate with a half-life of 20h. NOC-18 is widely used in *in vitro* and *in vivo* experiments investigating the effects of prolonged, constant NO delivery to cells and tissues. It is reported that NOC-18 at 100-200 μM releases 200-400 nM NO which is considered to be the pathological concentration of NO in tissue reported during inflammation and infection (Niedbala et al., 2007). The physiological concentration of NO range is between 100pM to 5nM. Depending on NO donors, the functional changes elicited by the generation of NO from iNOS can be comparable to what is observed with millimolar concentrations of NO donors under *in vitro* conditions (Katagiri et al ., 2003).

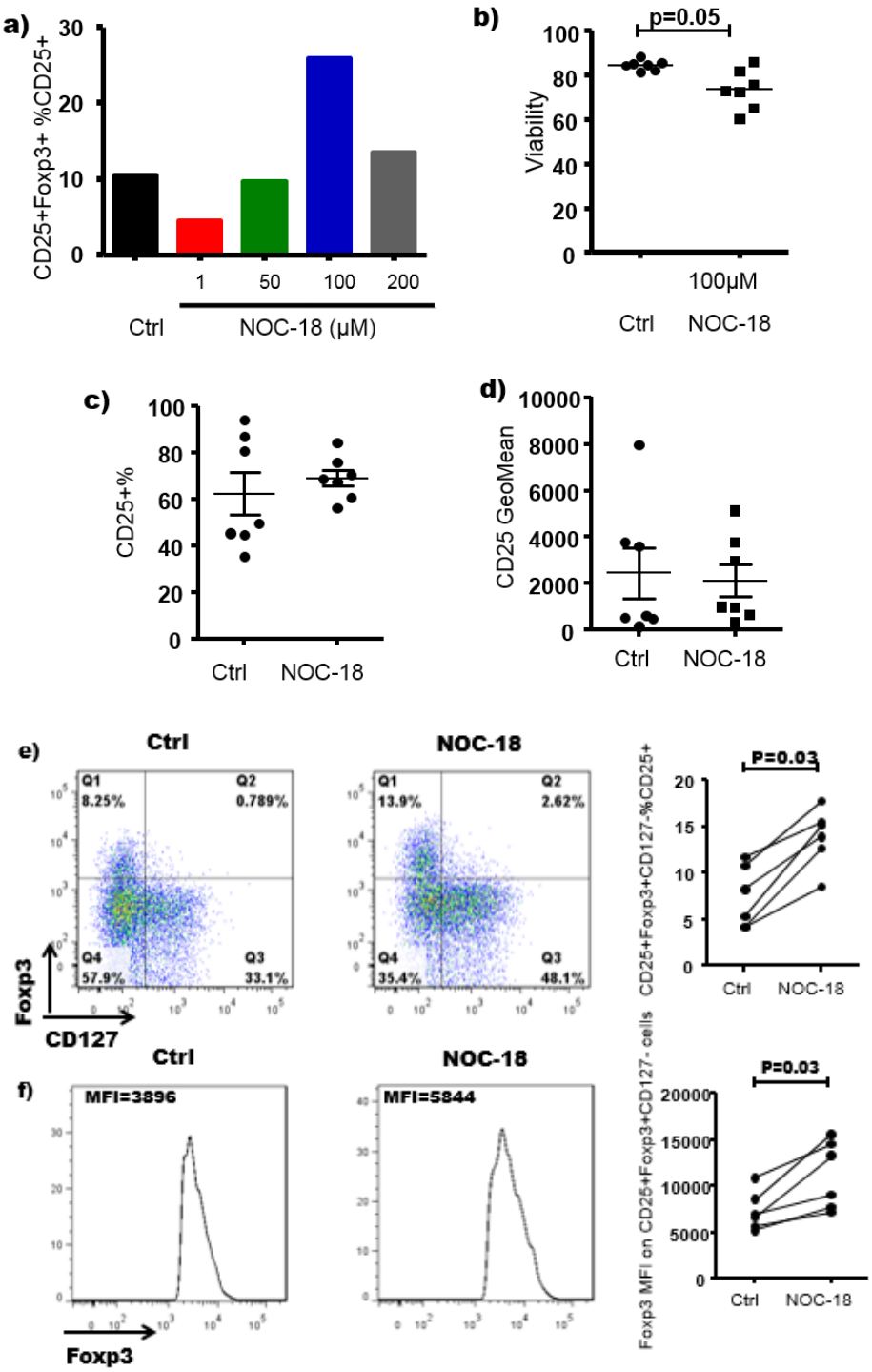
We first investigated the effect of NOC-18 on the phenotype of CD4+ T cells. To determine the optimal concentration for NOC-18, human peripheral blood CD4+ T cells were purified and cultured with plate bound anti-CD3 and anti-CD28 antibodies in the presence of different concentrations of NOC-18 for 5 days as described in Chapter 2.3.1.2. Activated CD4+ T cells treated with vehicle was used as control. The forkhead transcription factor Foxp3 is highly expressed on human CD25+ regulatory T cells and is critical for their development and function (Ziegler, 2006). Therefore, the expression of CD25 and Foxp3 were analysed by flow cytometry. Results showed that NOC-18 at 100 μM had the strongest inducing effect for CD25+Foxp3+ T cells. 100 μM NOC-18 did lead to a slight decrease in cell viability but this was of less than 10% indicating that the large majority of cells were still viable after 5 days culture in the presence of NOC-18 (Fig 3.1 a&b). Thus, NOC-18 at 100 μM was used for the following experiments.

As shown in Figure 3.1 c & d, neither the overall expression level (MFI) of CD25 nor the CD25+% were affected by NO indicating that T cell activation was not affected. Although FoxP3 protein is the most specific marker of Treg cells to date, studies have shown that Foxp3 is expressed in CD25low and CD25 negative CD4+ T cells and some CD8+ T cells (Ziegler, 2006). Activated effector T cells transiently express Foxp3, but at a level which is significantly lower than that on Tregs and is not associated with suppressive function. Other biomarkers are required to identify human Tregs. Foxp3 has been reported to interact with CD127 promoter and suppress the expression of CD127 on human Tregs (Liu, W., et al, 2006). CD4+CD127lo/- cells isolated from human peripheral blood remain anergic and suppress alloantigen responses *in vitro* (Liu et al., 2006). Therefore, the NO effect on CD25+Foxp3+CD127lo/- cells was analysed. As shown in Figure 3.1 e & f, the frequency of CD25+ Foxp3+ CD127- T cells and the MFI of Foxp3 on CD25+ Foxp3+CD127- T cells were significantly increased by NO, which indicated that NO promoted the development of cells with a conventional Treg phenotype. Cutaneous lymphocyte-associated antigen (CLA) is reported to be expressed on T cells that are specific for skin associated antigens and is considered as a skin homing marker (Bos et al., 1993). CLA expression on CD25+ Foxp3+ CD127- T cells was also significantly increased by NO (Fig 3.1 g). In support of this, the frequency of CD25+Foxp3+CD127- Tregs within the CLA+ CD4+ T cells was also increased by NOC18, while there was no significant effect of NO on the proportion of CLA+ T cell of total CD4+ T cells, suggesting that NO promoted differentiation of Tregs that were able to migrate to the skin (Fig 3.1 h&i).

To further confirm that the modulation of T cell phenotype was induced by NO released from NOC-18 and not caused by the backbone of the chemical, a different NO donating compound DETA-NO which has a similar half-life to NOC-18 was used. DETA-NO had the same effect

on the frequency of CD25+Foxp3+CD127- cells, and the level of Foxp3 and CLA expression as shown in Fig 3.1 j-l, which confirmed that the phenotype changes observed were most likely induced by NO.

To further investigate whether sustained release of NO was required for CD4+ T cell phenotype modulation, CD4+ T cell were activated and treated with 100 μM NOC-18. The culture medium containing 100 μM NOC-18 was taken out at different time points and fresh RPMI culture medium was added in to culture wells after the cells had been washed with PBS once. As shown in Fig 3.2 a&b, NO had to be present for at least 24 h to have effect on CD25+Foxp3+CD127- cell induction and CLA expression. Taken together, these results suggested that sustained exposure to NO at the levels reported in inflammation induced human Tregs that have enhanced ability to migrate to skin.


a)
CD25+Foxp3+ %CD25+
30
20
10
0
1
50
100
200
Ctrl
NOC-18 (μM)
b)
p=0.05
Viability
100
80
60
40
20
0
100μM
Ctrl
NOC-18
c)
CD25+%
100
80
60
40
20
0
Ctrl
NOC-18
d)
CD25 GeoMean
10000
8000
6000
4000
2000
0
Ctrl
NOC-18
e)
Ctrl
NOC-18
Q1
8.25%
Q2
0.789%
Q4
57.9%
Q3
33.1%
Q1
13.9%
Q2
2.62%
Q4
35.4%
Q3
48.1%
Foxp3
CD127
P=0.03
CD25+Foxp3+CD127-%CD25+
20
15
10
5
0
Ctrl
NOC-18
f)
Ctrl
NOC-18
MFI=3896
MFI=5844
Foxp3
P=0.03
Foxp3 MFI on CD25+Foxp3+CD127- cells
20000
15000
10000
5000
0
Ctrl
NOC-18

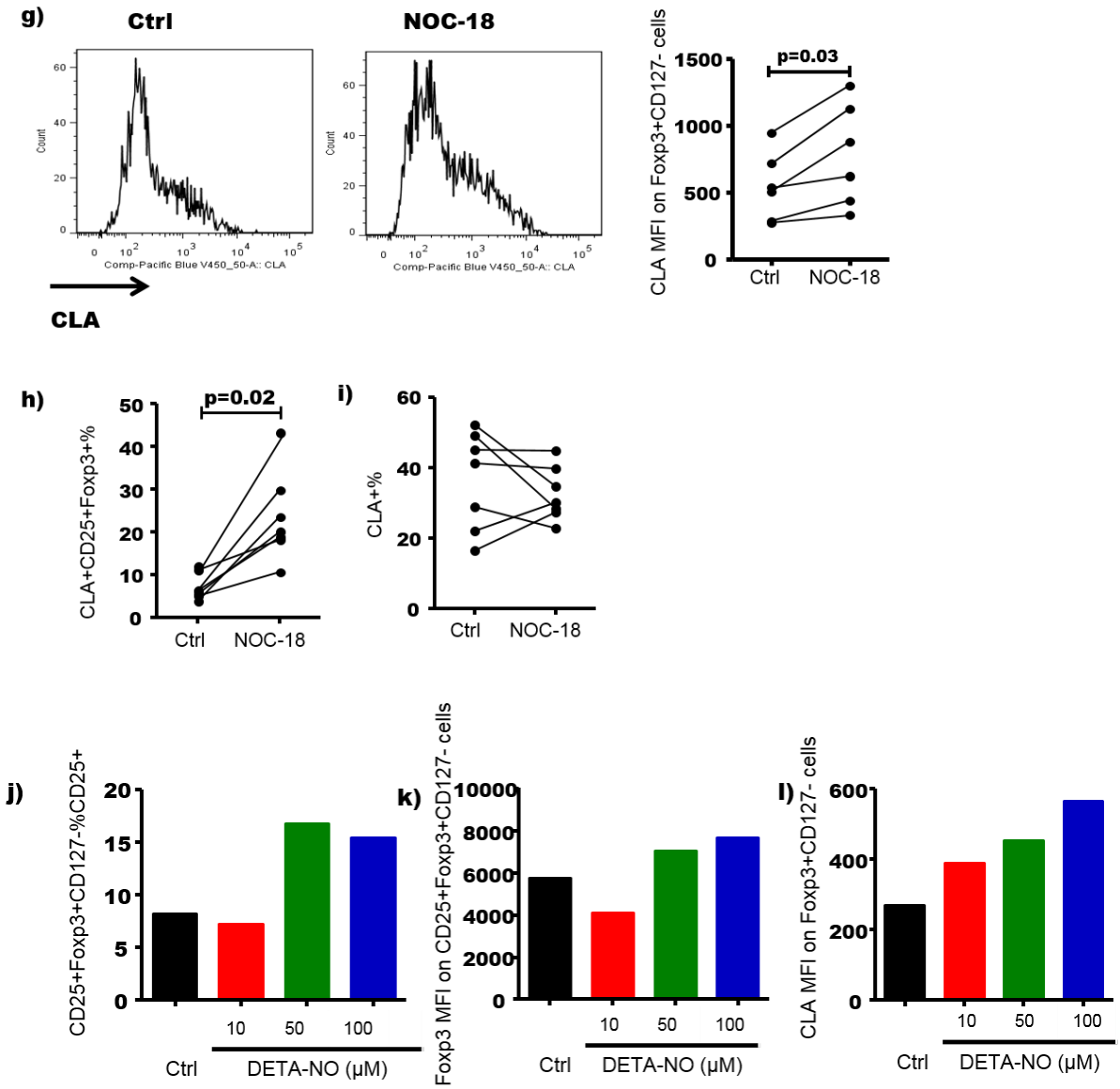


Fig 3.1 NO modulates human peripheral CD4+ T cell phenotype. Purified peripheral CD4+ T cells were activated with plate bound anti CD3/28 and treated with NO-donating compound for 5 days. T cell phenotype was measured by flow cytometry. a) Percentage of CD25+Foxp3+ cells of activated T cells after being treated with different concentrations of NOC-18. b) Viability of total cells after being treated with 100 μM NOC-18 (N=7). c-d) Geometric mean of CD25 expression and percentage of CD25+ cells on total CD4+ T cells (N=7). e-g) Percentage of CD25+Foxp3+CD127- cells of activated T cells, Foxp3 and CLA expression on CD25+Foxp3+CD127-T cell after total CD4+ T cells being treated with 100 μM of NOC-18 for 5 days with representative flow plots (N=6). h-i) Percentage of CLA+CD25+Foxp3+ CD127- cells and

CLA+ T cells of total cells after being treated with 100 µM of NOC-18 (N=6). j-l) Representative results of percentage of CD25+Foxp3+CD127- cells of activated T cells, Foxp3 and CLA expression on CD25+Foxp3+CD127-T cell after total CD4+ T cells being treated with 100µM of DETA-NO. Representative result of two separate experiments. P value was calculated using Wilcoxon matched pairs T test.

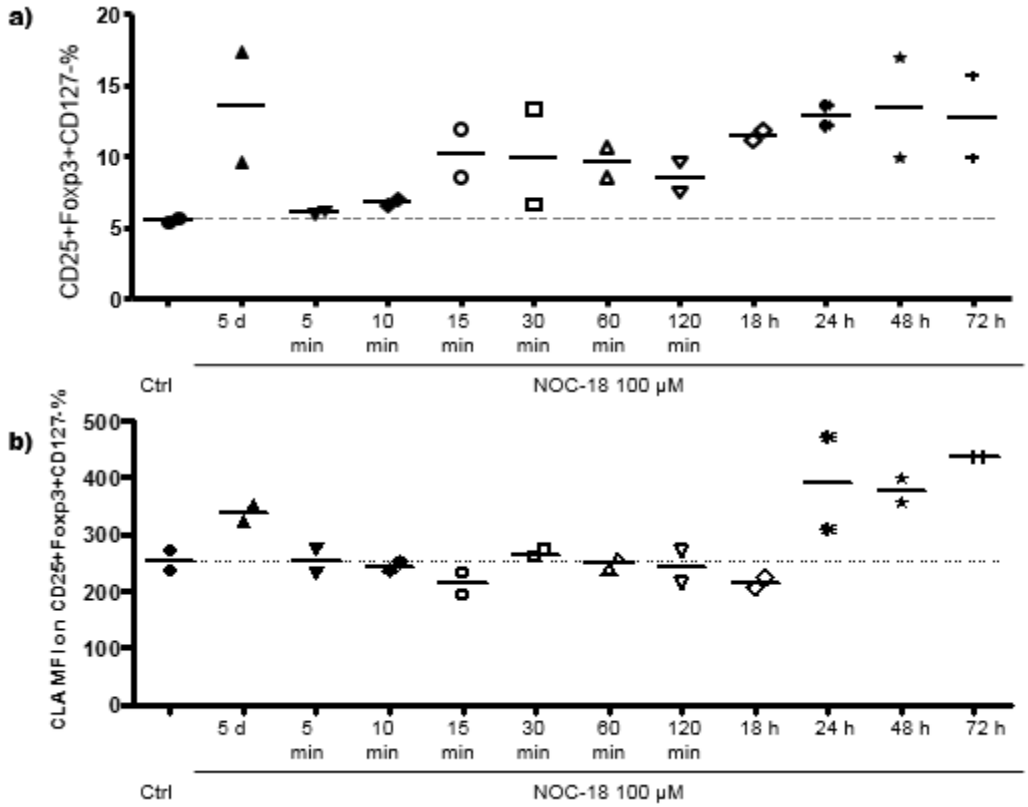


Fig 3.2 Sustained NO is required to modulate CD4+ T cell phenotype. Purified peripheral CD4+ T cells were activated with plate bound anti CD3/28 and treated with 100 µM NOC-18. NOC-18 containing medium was replaced with fresh culture medium at the indicated time points and T cell phenotype was measured by flow cytometry at Day5. CD4+ T cells that have been treated with100 µM NOC-18 for 5 days were used as positive control. a) Percentage of CD25+Foxp3+CD127- cells in the activated T cells (N=2). b) CLA expression on CD25+Foxp3+CD127- cells (N=2).

3.2.2 NO modulates CD4+ T cell phenotype via cGMP dependent pathways.

To investigate whether the effect of NO on CD4+ T cell phenotype depended on the sGC-cGMP signalling pathway, CD4+ T cells were cultured as above with 100 µM NOC-18 in the presence or absence of a specific competitive inhibitor of cGMP activation, 1-H-oxodiazolo-[1,2,4]- [4,3-a]quinoxalin-1-one (ODQ). Fig 3.3 showed that ODQ blocked the effect of NO on the frequency of CD25+ Foxp3+CD127- T cells in a dose dependent manner (Fig 3.3 a&b) These data demonstrated that NO modulated the phenotype of CD4+ Tcells via c-GMP dependent pathways.

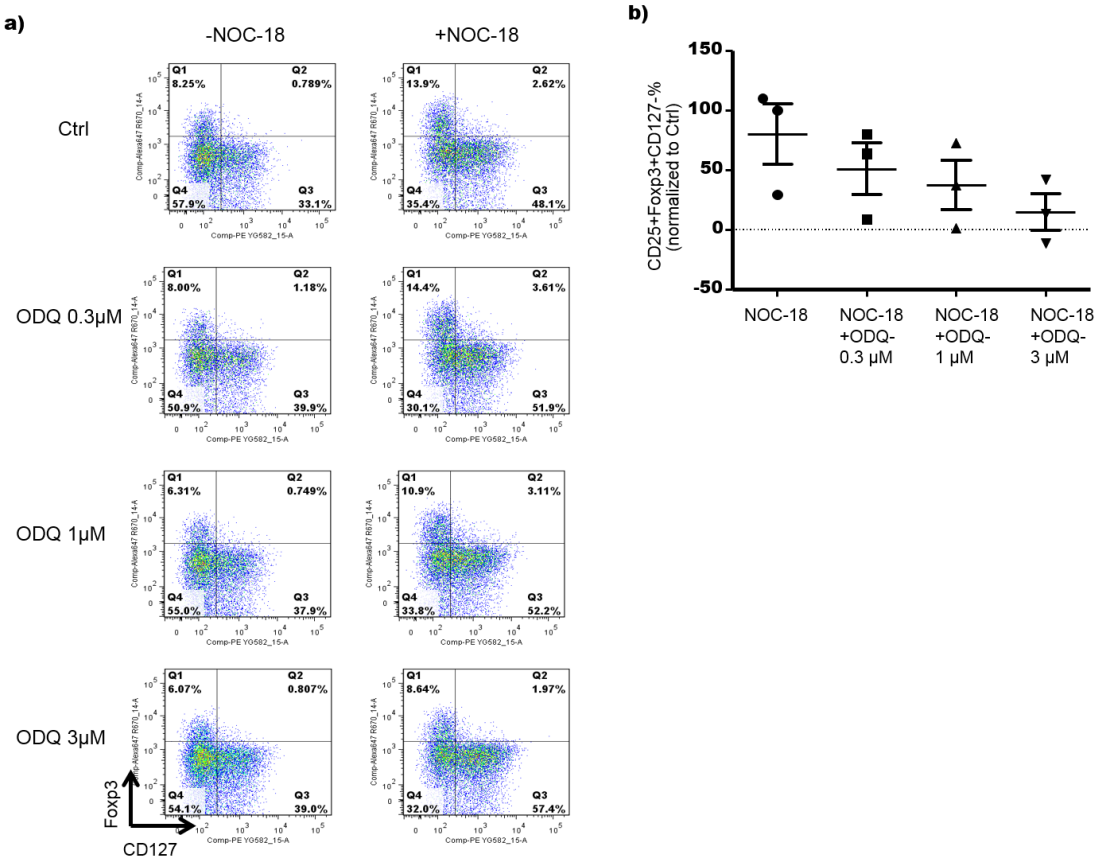


Fig 3.3 NO modulates CD4+ T cell phenotype via both cGMP dependent pathways. Purified peripheral CD4+ T cells were activated with plate bound anti CD3/28 and treated with 100 µM NOC-18 in the presence of different concentrations of ODQ. T cell phenotype was measured by flow cytometry at Day5. a) Representative FACS plot showing Foxp3 versus CD127 in activated T cells treated with or without

100µM NOC-18 in the presence of different concentrations of ODQ. b) Percentage of CD25+Foxp3+CD127- cells of activated T cells normalized to respective control (N=3).

3.2.3 NO induces CD25+ Foxp3+ CD127- T cells from CD4+CD25lo T cells.

It has previously been shown that NO inhibited proliferation of CD4+ T cells (Bogdan, C, 2001). Therefore, it was possible that the increased frequency of CD25+ Foxp3+ CD127- T cells reflected decreased expansion of Foxp3- T cells. To assess this possibility, the proliferation of Foxp3+ and of Foxp3- cells was assessed. CD4+ T cells were stained with the proliferation dye ef670 before being activated and treated with 100µM NOC-18 for 5 days as described in Chapter 2.3.2.4. Proliferation and phenotype of CD4+ T cells were analysed by flow cytometry. As shown in Fig 3.4a, NO inhibited the proliferation of total CD4+ T cells as reported before although it did not reach significant. However, proliferation of the Foxp3+ cells was inhibited to a similar extent as the Foxp3- fraction. No significant difference in proliferation was observed between the Foxp3- and Foxp3+ fractions (Fig 3.4b) indicating that the increased frequency of CD25+ Foxp3+ CD127- T cells was not caused by a decreased number of CD25- T cells.

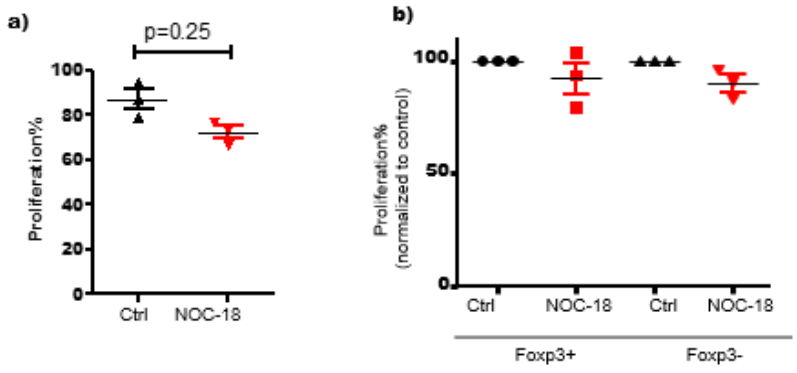


Fig 3.4 NO inhibits Fopx3+ and Foxp3- cell proliferation to a similar extend. Purified CD4+ T cells were stained with proliferation dye ef670 before being activated with plate bound anti-CD3/28 and treated

with 100 μM NOC-18 for 5 days. T cells proliferation and phenotype was analysed by flow cytometry. a) Proliferation of total CD4+ T cells after being treated with 100 μM NOC-18 for 5 days. b) Proliferation of Foxp3+ and Foxp3- cell population after being treated with 100 μM NOC-18 for 5 days.

To further confirm the effect of NO on Treg proliferation in our model, CD4+ T cells were labelled with ef670and activated with anti-CD3/28 in the presence of 100 μM NOC-18, and Foxp3 expression and proliferation of CD4+T cells were analysed. As shown in Fig 3.5, by day 5, there was a marked increase in the proportion of Foxp3+ cells and, importantly, the majority of these had lower ef670 staining, confirming a close correlation between Foxp3 enrichment and proliferation.

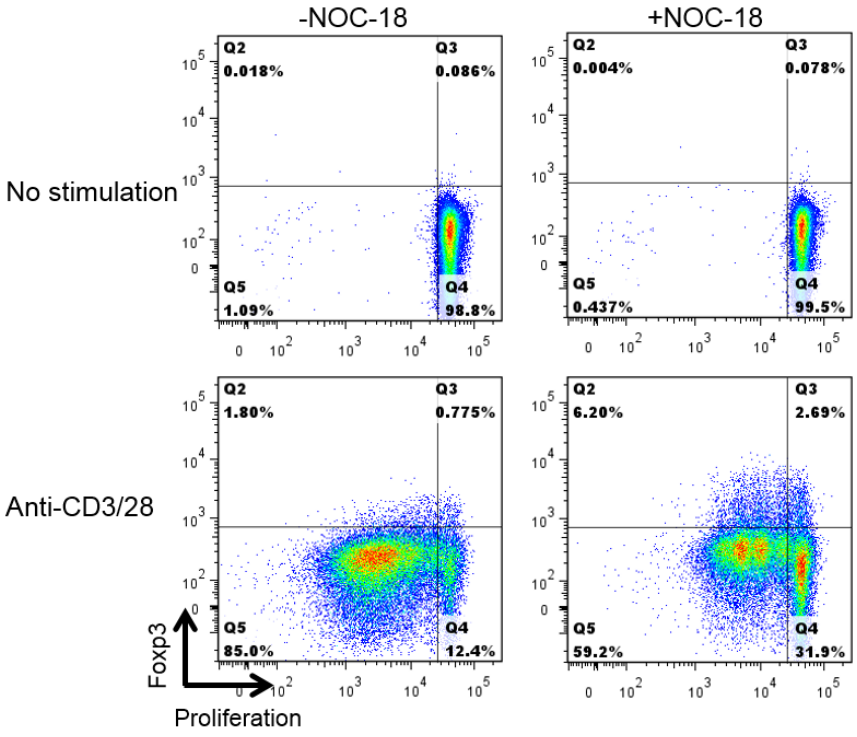


Fig 3.5 Increased frequency of CD25+ Foxp3+ CD127- T cells involves Foxp3+ T cells proliferation. CD4+ T cells were stained with the proliferation dye ef670 before being activated with plate bound anti-

CD3/28 and treated with 100μM NOC-18 for 5 days. Foxp3 expression and T cell proliferation were analysed by flow cytometry. Representative FACS plot showing Foxp3 versus proliferation of total CD4+ T cells at Day 5 (N=3).

These data suggested that NO could either promote the proliferation of pre-existing nTreg, or induce expression of Foxp3 in Foxp3- CD4+ T cells, or both. To check whether NO induced expansion of Foxp3+ nTreg circulating in blood leading to increased frequency of CD25+Foxp3+CD127- T cells, CD4+CD25hi cells were isolated from total CD4+ T cells and cultured in the presence of 100μM NOC-18 for 5 days as described in Chapter 2.2.5. After enrichment, CD4+CD25hi % was increased to 48.2% compared with 4.5% in total CD4+ T cells as shown in Fig 3.6a. Although the percentage of CD25+Foxp3+CD127- T cells was also increased by NO from CD4+CD25hi cells (Fig 3.6b), it was difficult to conclude where the Foxp3+ cells came from since the purity for the CD4+CD25hi cells was only ~30%.

We next asked if the increased frequency of CD25+Foxp3+CD127- T cells was induced from the CD25lo T effector cell population. Therefore, CD25hi cells were depleted from the total CD4+ T cells (Fig 3.6c) and the CD25lo cells were cultured with 100μM NOC-18 for 5 days. As shown in Fig 3.6d, CD25+Foxp3+CD127- cells were induced from CD25lo cells after being activated for 5 days. NO significantly increased the frequency of CD25+Foxp3+CD127- cells from activated CD25lo cells and expression of CLA was also increased by NO on the induced Foxp3+ T cells (Fig 3.6 d&e). Taken together, these results suggested that NO promoted CD25+ Foxp3+ CD127- T cells differentiation from CD4+CD25lo T effector cells.

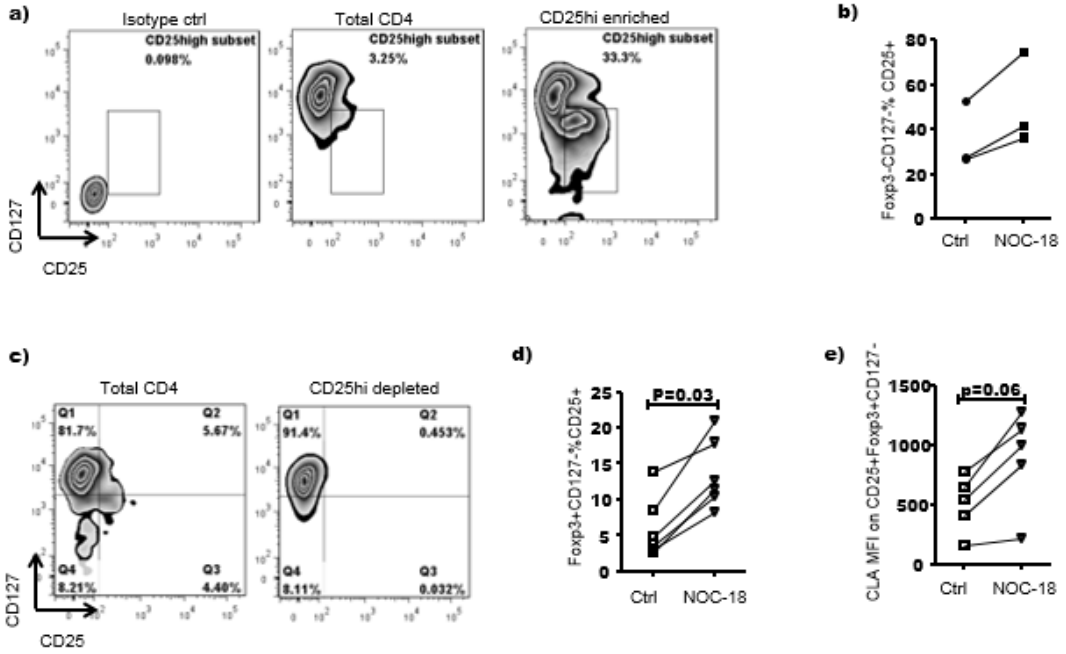


Fig 3.6 NO promotes CD25+ Foxp3+ CD127- T cells differentiation from CD4+CD25lo T cells. CD25hi and CD25lo cells were isolated from peripheral CD4+ T cells and were activated and cultured with 100μM NOC-18 for 5 days. T cells phenotype was analysed by flow cytometry. a) Representative FACS plot showing CD25 versus CD127 of total CD4+ T cell and CD25hi cells before and after enrichment. b) Percentage of CD25+Foxp3+CD127- cells of activated T cells after CD25hi T cells being treated with NOC-18 for 5 days (N=3). c) Representative FACS plot showing CD25 verses CD127 of total CD4+ T cell and CD25lo cells before and after depletion. d-e) Percentage of CD25+Foxp3+CD127- cells of activated T cells (N=6) and CLA expression on CD25+Foxp3+CD127- cells after CD25lo T cells (N=5) being treated with NOC-18 for 5 days. P value was calculated using Wilcoxon matched pairs T test.

3.2.4 NO induced CD25+Foxp3+CD127- cells have immune-regulatory function.

To next assess whether CD25+Foxp3+CD127- cells induced by NO have immune-regulatory function, I performed functional suppression assays. CD4+ T cells were divided into two parts immediately after isolation. One part was activated with anti-CD3/28 and treated with

100μM NOC-18 for 5 days, which were used as suppressor cells in the following experiments (NO-CD4). The other part was frozen in -80 degree on the same day of isolation. After 5 days, the frozen cells were thawed, stained with proliferation dye ef670 and used as responder cells, which were cultured together with unlabelled suppressor cells at different ratios. Based on staining of ef670, the responder cells could be separated from the suppressor cells, and their proliferation was analysed by flow cytometry.

As shown in Fig 3.7a and b, 71.8% of responder cells proliferated when they were cultured alone without suppressor cells. Based on the results shown in Fig 3.1, approximately 20% of the total CD4+ cells were CD25+Foxp3+CD127- after being cultured with 100μM NOC-18 for 5 days. Therefore, in order to achieve a responder cell : Foxp3+ cell ratio of 1:1 and 1:2, responder cells were mixed with total CD4+ T cells that were cultured with NOC-18 for 5 days (NO-CD4) at ratios of 1:5 and 1:10. When the responder cells were co-cultured with NO-CD4 at 1:5, their proliferation decreased and further decreased when the responders and NO-CD4 ratio increased to 1:10 (Fig 3.7a and b). Thus NO-CD4 T cells significantly inhibited the proliferation of autologous CD4+ T cell in a dose-dependent manner. To confirm that suppression of proliferation was not due to nutrient deprivation caused by increased number of cells cultured in one well, control CD4+ T cells that had been activated for 5 days without NOC-18 were co-cultured together with responder cells and proliferation of the responder cells were analysed. As shown in Fig 3.7c, the proliferation of responder cells were increased when co-cultured with activated CD4+ T cells at 1:5 ratio. The proliferation of responder cells were slightly decreased by activated CD4+ T cells at 1:10 ratio although the changes were relatively small compared with that caused by NO-CD4. The expansion of existing nTreg in CD4+ T cells might contribute to this effect which also indicated that Treg after freeze-thraw cycle still have suppressive function.

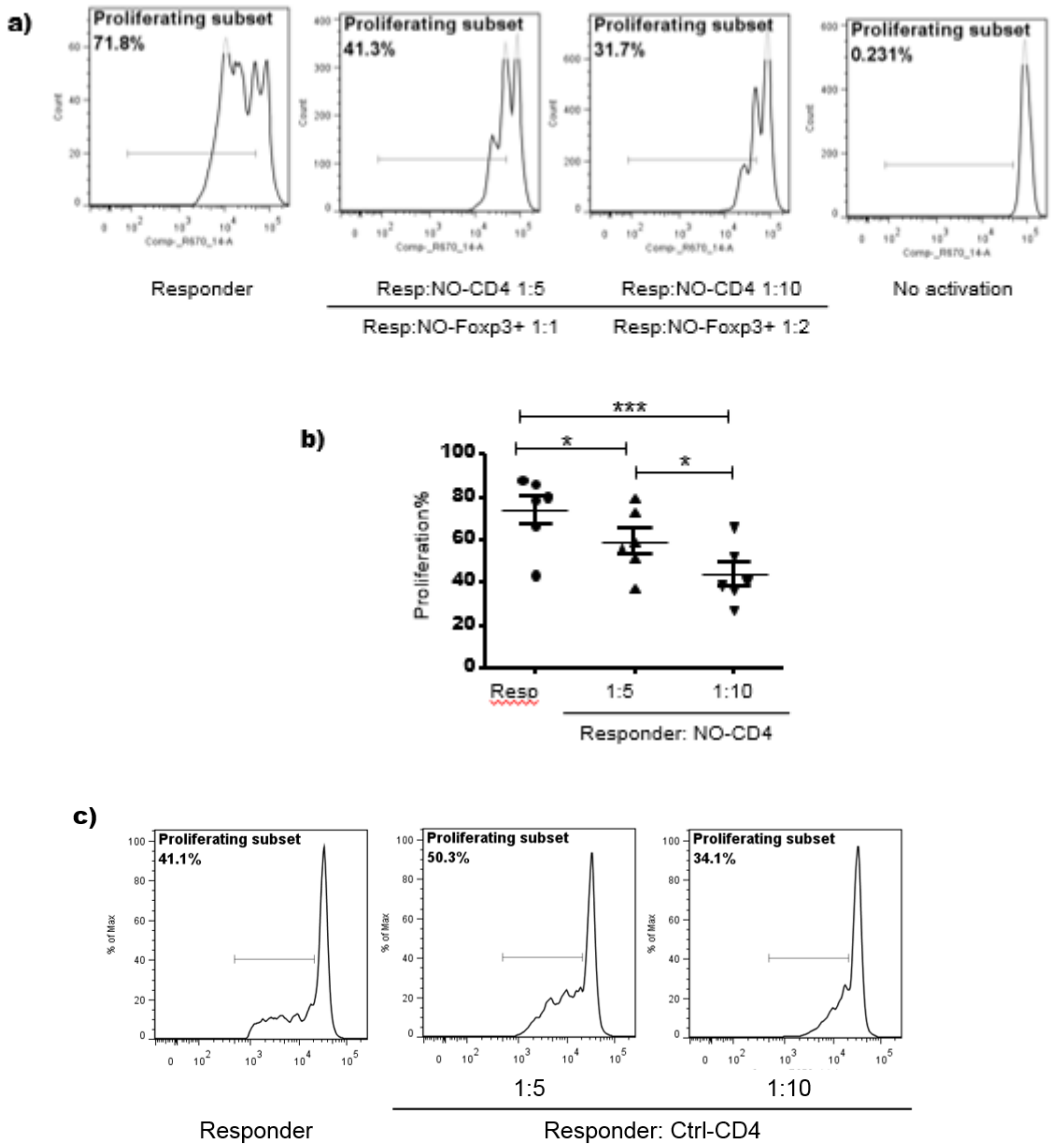


Fig 3.7 NO-CD4 T cells significantly inhibit the proliferation of autologous CD4+ T cell in a dose-dependent manner. Purified CD4+ T cells were activated with plate bound anti-CD3/28 and cultured with 100 µM NOC-18 for 5 days (NO-CD4). Autologous CD4+ T cells were used as responder cells and cultured with NO-CD4+ T cells at different ratios for 4 days. Proliferation of responder cells was analysed by flow cytometry. a) Representative FACS plot showing proliferation of responder cells cultured with NO-CD4 T cells. b) Proliferation of responder cells cultured with NO-CD4 T cells at different ratios at Day4 (N=6) c) Representative FACS plot showing proliferation of responder cells cultured with total CD4+ T cells that have been activated with plate bound anti-CD3/28 for 5 days. Representative result of two separate

experiments. P value was calculated by Friedman test followed by Dunns post test.

To further confirm that CD25+Foxp3+CD127- cells within the NO-CD4 cells were responsible for the suppressive function, CD25hi cells (NO-CD25hi) were isolated from CD4+ T cells that have been activated and treated with 100μM NOC-18 for 5 days as described in Chapter 2.2.5. Purity of NO-CD25hi cells was analysed after isolation, which showed that NO-CD25hi cells also expressed high level of Foxp3 (Fig 3.8 a). NO-CD25hi cells were used as suppressor cells and co-cultured with responder cells at different ratios. Compared with NO-CD4 T cells, NO-CD25hi showed higher efficiency in inhibiting autologous CD4+ T cell proliferation, while the proliferation of responder cells was inhibited more than 50% when responder and NO-CD25hi were cultured at 2:1 ratio (Fig 3.8b). Taken together, these results indicated that NO induced functional Foxp3+ regulatory T cells in total CD4+ T cells and in CD4+CD25- T cells.

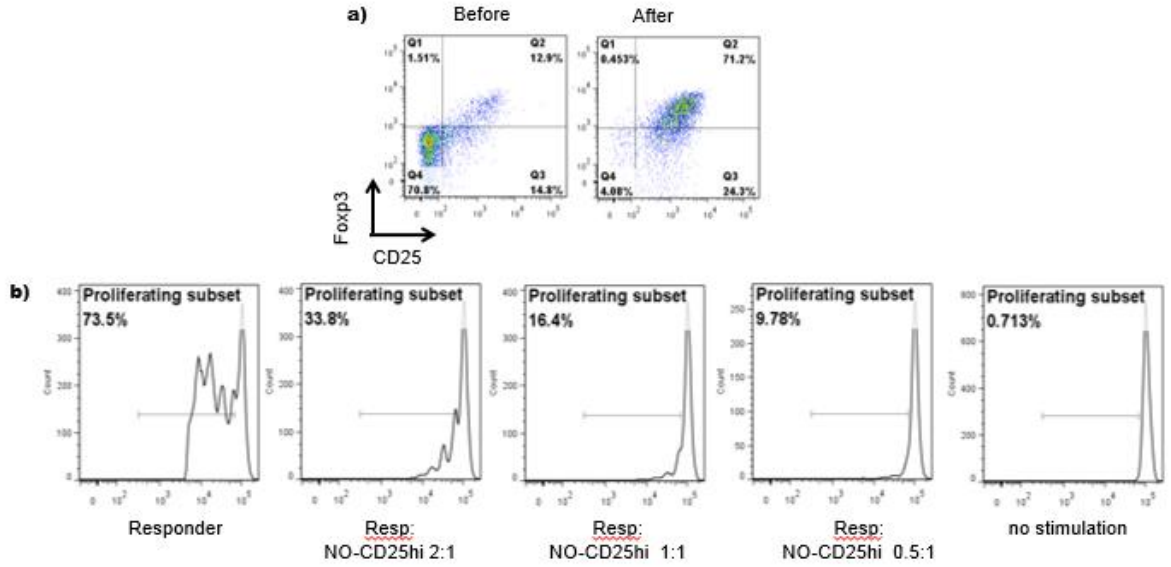


Fig 3.8 NO induced CD25+Foxp3+CD127- cells inhibit proliferation of autologous CD4+ T cell in a dose-dependent manner. CD25hi cells were isolated from CD4+ T cells that were activated with anti-

CD3/28 and treated with 100μM NOC-18 for 5 days (NO-CD25hi). NO-CD25hi cells were co-cultured with autologous CD4+ T cells at different ratios for 4 days and proliferation of autologous CD4+ T cells were analysed by flow cytometry. a) Representative FACS plot showing CD25 verses Foxp3 of NO-CD4 T cells and NO-CD25hi cells before and after isolation. b) Proliferation of responder cells cultured with NO-CD25hi T cells at different ratios at Day 4. Representative result of 6 separate experiments.

3.2.5 Immuno-suppression of NO-induced Foxp3+Treg depends on cell-cell contact.

It has been reported before that cell-cell contact plays a non-redundant role in Treg mediated immune suppression. To assess whether NO-induced Foxp3+Tregs exert their regulatory function through cell-cell contact, I performed transwell experiments. As described in Chapter 2.3.2.6, responder cells were seeded at bottom of 24-well plate and activated with anti-CD3/28. Total CD4+ T cells that had been treated with 100μM NOC-18 for 5 days (NO-CD4) were used as suppressor cells in this experiment and they were cultured in the upper chamber of the transwell. As shown in Fig 3.9 a&b, NO-CD4 Tcells inhibited the proliferation of responder cells as showed above. The use of transwells prevented the suppressive function of NO-CD4 on proliferation indicating that NO-induced Foxp3+ Treg required cell-cell contact for their regulatory function.

Besides cell-cell contact, cytokines with suppressive functions such as IL-10 derived from Tregs have been implicated as important mediators of the suppressive function of Tregs. Therefore, the concentrations of the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine IFNγ in the cell culture supernatants from CD4+ T cells cultured with or without 100μM NOC-18 were determined by ELISA. As expected, both IL-10 and IFNγ secretion were decreased by NO since the proliferation of CD4+ T cells were inhibited by NO. The ratio of IL-10/ IFNγ was decreased by NO as well (Fig3.10 a,b,c).

To further investigate the potential role of suppressive cytokines in NO-CD4 mediated immune suppression, culture supernatants from CD4+ T cells cultured with or without 100 μM NOC-18 for 5 days were mixed with fresh RPMI at different ratios and the mixtures were added to cultures of ef670-labeled responder T cells and proliferation of responder cells was analysed by flow cytometry. In contrast to the results obtained using the transwells, the proliferation of responder cells was not affected by the culture supernatants (Fig 3.10 d & e). These data suggested that the cytokines secreted by NOC-18 treated CD4+ T cell were not sufficient for NO-CD4 mediated immune suppression.

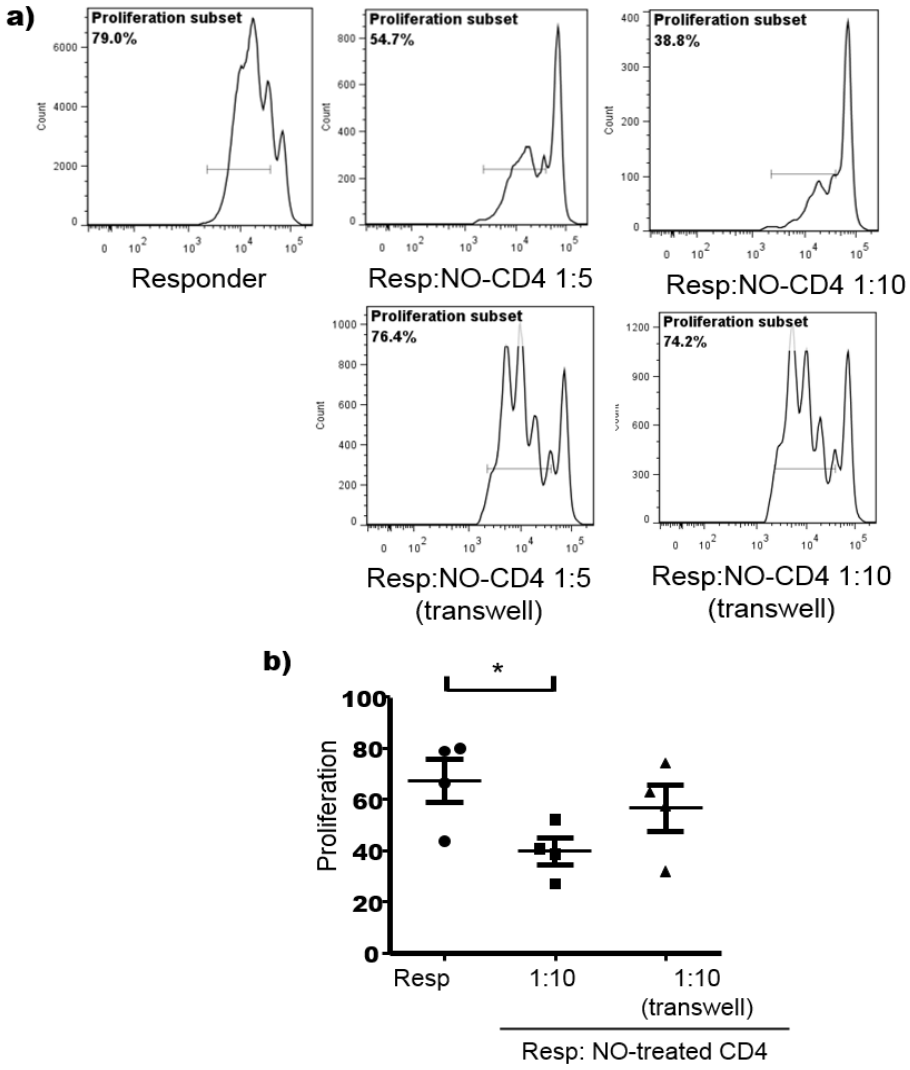


Fig 3.9 NO-induced Foxp3+ Tregs require cell-cell contact for its suppressive function. Purified CD4+ T cells were activated with anti-CD3/28 and treated with 100μM NOC-18 for 5 days (NO-CD4), which were used as suppressor cells. Suppressor cells were seeded either together with autologous CD4+ T cells or seeded in the upper well of transwell to be physically separated from autologous CD4+ T cells. Proliferation of autologous CD4+ T cells were analysed by flow cytometry. a) Representative FACS plot showing proliferation of autologous CD4+ T cells cultured with NO-CD4 at different ratios with or without transwell for 4 days. b) Proliferation of autologous CD4+ T cells after being co-cultured with NO-CD4 at 1:10 ratio in the presence or absence of transwell for 4 days (N=4). P value was calculated by Friedman test followed by Dunns post test.

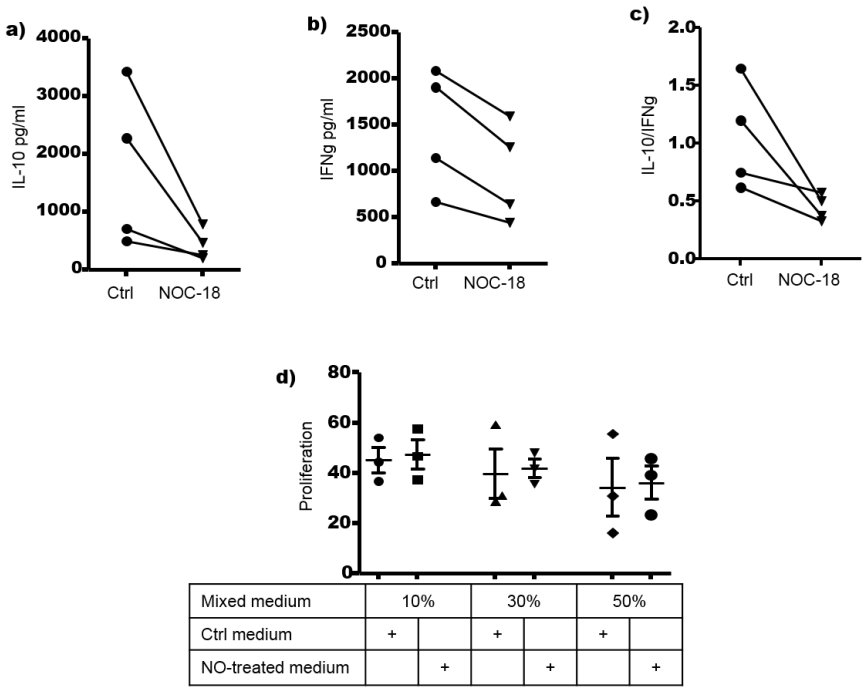


Fig 3.10 IL-10 secreted by NO-induced Foxp3+ Tregs is not sufficient for their suppressive function. Culture supernatants from CD4+ T cells cultured with or without 100 μM NOC-18 for 5 days were mixed with fresh RPMI at different ratios and the mixtures were transferred to ef670-labeled autologous CD4+ T cells. Proliferation of autologous CD4+ T cells was analysed by flow cytometry. a-c) IL-10 and IFNγ concentration and IL-10/ IFNγ ratio in the culture supernatant of CD4+ T cells being activated with CD3/28 and treated with 100 μM NOC-18 for 5 days (N=4). d) Proliferation of autologous CD4+ T cells after being cultured using mixed culture medium for 4 days.

3.2.6 Multiple pathways mediate the immuno-regulatory function of NO-induced Foxp3+Treg.

Apart from IL-10 signalling, multiple signalling pathways have been reported to be utilized by Foxp3+Tregs to exert their regulatory function. The most commonly reported of these are membrane bound TGFβ, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1). To further assess whether any of these receptors mediated NO-induced

Foxp3+Tregs suppressive function, I blocked each of these pathways using either chemical agents or blocking antibodies. I used the compound SB-431542 (SB), a competitive ATP binding site kinase inhibitor that inhibits TGFβ signalling pathway, and antibodies that block PD-1 and its ligand PD-L1 and CTLA-4. Purified CD4+ T cells were activated with plate bound anti-CD3/28 and treated with 100 μM NOC-18 for 5 days to generate NO-Foxp3+Treg. CD25hi cells (NO-CD25hi) were isolated and co-cultured with autologous CD4+ T cells for 4 days in the presence or absence of the compound/antibodies mentioned above. The proliferation of autologous CD4+ T cell was then analysed by flow cytometry.

As shown in Fig 3.11a, NO-CD25hi cells inhibited the proliferation of autologous CD4+ T cells as expected. The inhibition was partially prevented in the presence of the TGFβ signalling inhibitor SB. Antibodies blocking PD-1/PD-L1 also partially reversed the inhibitory effect of NO-CD25hi on the proliferation of autologous CD4+ T cells. However, neither of these pathways could fully reverse the inhibitory effect of NO-CD25hi. Therefore, to test whether there was any synergistic effect of these pathways, autologous CD4+ T cells were co-cultured with NO-CD25hi while both TGFβ and PD/PD-L1 signalling pathways were blocked. No synergistic effect was seen when both pathways were blocked (Fig 3.11a). As shown in Fig 3.11b, blocking CTLA-4 did not affect the inhibitory function of NO-CD25hi. Taken together, these data suggested that both TGFβ and PD-1 signalling pathways were independently involved in mediating the immunosuppressive function of NO induced Foxp3+Tregs but that neither were sufficient to account for the entire suppression seen. Other unidentified pathways must also be involved.

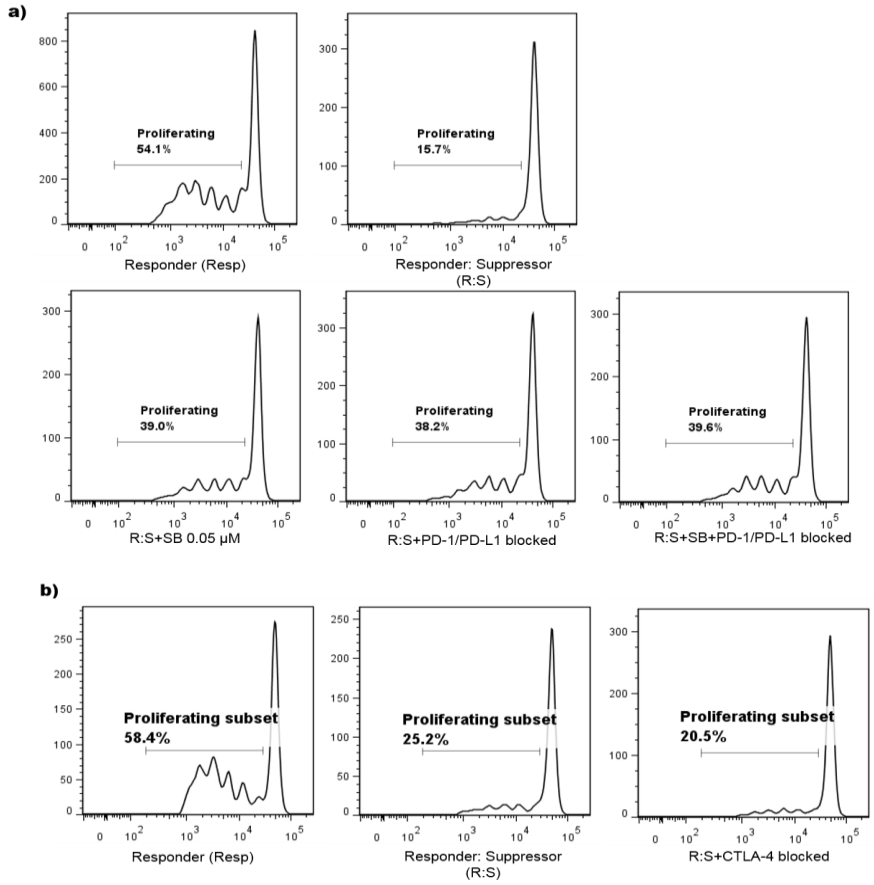


Fig 3.11 Multiple pathways mediate immune-regulatory function of NO-induced Foxp3+Treg. CD25hi cells (NO-CD25hi) were isolated from total CD4+ T cells that have been activated and treated with 100 µM NOC-18 for 5 days. NO-CD25hi were co-cultured with autologous CD4+ T cell for 4 days in the presence of TGFβ signalling inhibitor SB, antibodies blocking PD-1 and its ligand PD-L1 and CTLA-4. Proliferation of autologous CD4+ T cells were analysed by flow cytometry. a) Representative FACS plot showing proliferation of autologous CD4+ T cells cultured with NO-CD25 at Day4 in the presence of TGFβ signalling inhibitor SB (representative result of 6 separate experiments).), antibodies blocking PD-1 and its ligand PD-L1(representative result of 6 separate experiments.) or together (representative result of 3 separate experiments.). b) Representative FACS plot showing proliferation of autologous CD4+ T cells cultured with NO-CD25 at Day4 in the presence antibodies blocking CTLA-4. Representative result of 3 separate experiments.

3.2.7 NO modulates differentiation of human monocyte-derived dendritic cells (mo-DCs).

DCs have unique capacity to process antigen, present it to CD4+ T cell and shape the differentiation of naive CD4+ T cells. To investigate whether NO could affect CD4+ T cell differentiation indirectly through affecting DC function, I analysed the effect of NO on human mo-DCs differentiation and maturation. CD14+ monocytes were isolated from PBMCs and cultured with GM-CSF and IL-4 for 6 days to allow them to differentiate into immature mo-DCs as described in Chapter 2.3.1.1. The immature mo-DCs were matured in the presence of Poly I/C for 48 h and the phenotype of both immature and mature mo-DCs was analysed by flow cytometry. Compared with immature DCs which expressed medium level of CD86 and low level of CD80, matured mo-DCs expressed high levels of CD80 and CD86 indicating that the protocol used to differentiate and maturate mo-DCs worked (Fig 3.12 a). As shown in Fig 3.12 b, NO significantly decreased the expression of antigen-presenting molecule HLA-DR and slightly decreased the expression of co-stimulatory molecule CD86 on immature mo-DCs as previously reported (Bogdan, C, 2001). The other markers tested on immature mo-DC were not affected by NO. None of the markers analysed were modified by NO on mature mo-DCs (Fig 3.12 c). Taken together, these results indicated that NO may affect the differentiation of mo-DCs which indicated that NO may also affect CD4+T cell differentiation indirectly.

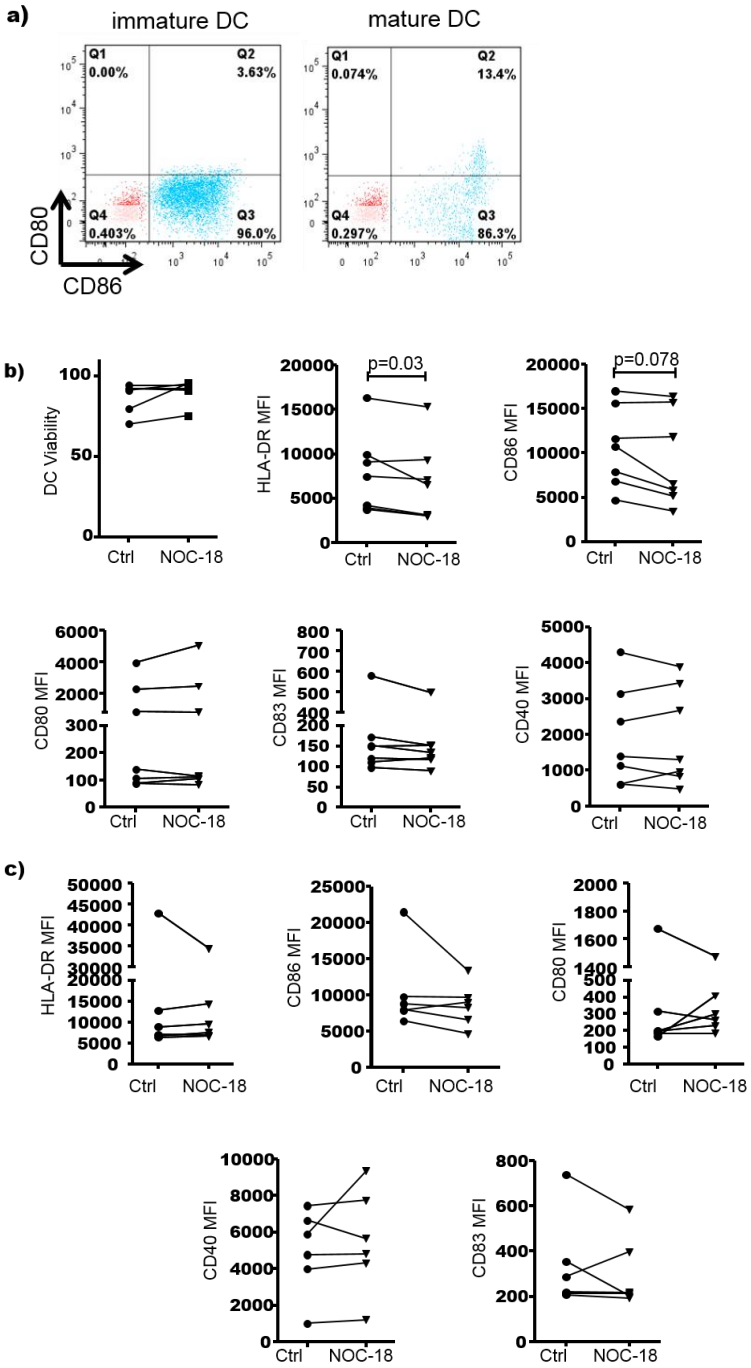


Fig 3.12 NO modulates differentiation of human monocyte-derived dendritic cells (mo-DCs). CD14+ monocytes were isolated from PBMCs and cultured with cytokines including GM-CSF and IL-4 for 7 days with or without 100 µM NOC-18 to generate immature mo-DCs. The immature mo-DCs were matured Poly I/C for 48 h and phenotype of both immature and mature mo-DCs were analysed by flow cytometry. a) Representative FACS plot showing CD80 verses CD86 of both immature and mature mo-DCs. b) Viability

and expression of antigen-presenting molecules, costimulatory molecules and maturation molecules on immature mo-DCs (N=7). c) Expression of antigen-presenting molecules, costimulatory molecules and maturation molecules on mo-DCs matured with Poly I/C for 48h (N=6). P value was calculated using Wilcoxon matched pairs T test.

3.3 Discussion

Extensive studies have revealed the substantial heterogeneity of Tregs in both mice and humans. In addition to nTregs, Tregs can be induced from CD4+CD25- T cells by specific antigens in humans and mice. Several other types of Tregs have also been reported such as type 1 Tregs which do not express Foxp3 and mediate their suppressive function through IL-10, whereas Tregs induced by TGF-β modulate immune responses by secreting TGF-β and IL-10 (Sakaguchi et al., 2008). Previous murine studies have shown that a new type of Treg (NO-Treg) was induced from CD4+CD25- T effector cells which was CD4+CD25-, Foxp3-, GITR+, CD27+, with a Th2 phenotype and the ability to ameliorate inflammatory diseases in a contact dependent and IL-10 dependent manner. The induction of NO-Treg did not involve NO-cGMP signalling pathway (Niedbala et al., 2007).

Here, I have shown that NO modulated the phenotype and function of human peripheral CD4+ T cells, which was distinct from its reported effect on murine CD4+ T cells in several important aspects. The cell population induced by NO from human peripheral total CD4+ T cells and CD4+CD25- effector T cells was CD25+ Foxp3+ CD127-, which phenotypically represented Tregs. This effect could be blocked by a selective soluble guanylate cyclase (sGC) inhibitor indicating the involvement of cGMP. NO also affected CLA expression on CD25+Foxp3+CD127- cells which may affect the skin-homing ability of these cells. NO-induced CD25+ Foxp3+ CD127- cells inhibited the proliferation of autologous CD4+ T cells in a contact dependent manner, the effect of which was mediated by membrane bound TGFβ and PD-1/PD-L1; whereas anti-inflammatory cytokines secreted by NO treated CD4+ T cells were not sufficient. This was further confirmed by analysing cytokines in the culture supernatant from NO treated CD4+ T cells in which NO decreased IL-10 and led to a decreased IL-10/IFNγ ratio. Taken together, these data suggested that NO induced functional

Foxp3+Tregs from human peripheral CD4+ T cells.

It is reported that 100μM of NOC-18 slowly releases 200nM NO with a half-life of 20h. The 200nM dose of NO used here is likely to occur *in vivo* in sites of inflammation and has been used routinely in mouse *in vitro* experiments. During inflammation, NO could be produced locally at the site of inflammation or in the lymphoid organs as a consequence of the production and stimulatory effects of pro-inflammatory cytokines (such as IFNγ). Foxp3+Tregs induced by NO may control inflammation by reducing proliferation of effector T cells and possibly the secretion of pro-inflammatory cytokines, which forms a negative feedback loop.

It is well known that NO inhibits endothelial activation and surface expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, in human endothelial cells stimulated with inflammatory cytokines. The expression of E-selectin on dermal endothelial cells has been proposed to be important in skin inflammatory responses (Sakaguchi et al., 2008). Previous study has shown that a nitric-oxide derivative of prednisolone reduced leukocyte adherence to endothelial cells in colitis mouse model which contributed to its anti-inflammatory effect (Fiorucci, Antonelli et al. 2002). The importance of migratory behaviour of Tregs in controlling inflammation has been reported before. In a murine model of CHS, UV radiation-induced Tregs could only inhibit the induction but not the elicitation phase of CHS when injected i.v into sensitised mice due to the lack of ability of Tregs to migrate to the skin (Schwarz el al., 2004). Thus, increased expression of CLA on NO-induced CD25+Foxp3+CD127-Tregs from human peripheral CD4+ T cells could compensate for the decreased expression of its ligand E-selectin on endothelial cells caused by NO.

In a murine model of MS, NO-Treg have been shown to be effective in treating established inflammation (Niedbala et al., 2013), demonstrating that Tregs generated *ex vivo* have significant potential as cellular therapeutics. Therefore, understanding the underlying mechanisms responsible for NO-induced Treg generation from peripheral human CD4+ T cells will have potential influence in optimizing protocols for clinical use. Although it is well known that NO inhibits proliferation of CD4+ T cells, no previous studies have shown whether NO selectively inhibits effector T cell or Treg proliferation. Here we have shown that proliferation of both effector T cells and Tregs were inhibited by NO to a similar extend, which suggested that the enrichment of CD25+Foxp3+CD127- Treg cells could not be explained by decreased proliferation of non-Treg cells or selective expansion of Treg. Alternatively, a previous murine study reported that NO induced by exogenous IFN-γ from allogeneic GM-CSF/TGF-β-conditioned immature DC resulted in overall selection for Tregs via proliferation and conversion of non-Treg precursors (Feng et al., 2008). In keeping with this, the majority of NO-induced Foxp3+ cells were proliferating cells. However, it is not possible to determine whether the enrichment shown in Fig 3.5 is due to direct conversion of Foxp3– cells or expansion of a small starting population of Foxp3+ cells contained in the total CD4+ T cell. To test if NO converts circulating non-Treg cells to cells with a Treg phenotype, nTreg depleted CD25lo/- T cells were activated and cultured in the presence of NO. NO induced CD25+Foxp3+CD127- cells, indicating that NO is able to promote differentiation of CD25+Foxp3+CD127- cell from CD25lo/- T cells. I also tested whether NO promotes expansion of Foxp3+ nTregs circulating in blood by isolating CD25hi T cells from total CD4+ T cells. Due to the purity issue of the CD25hiCD127lo/- cells isolated from peripheral CD4+ T cells, the possibility that NO induced the expansion of nTreg within the blood could not be ruled out. Despite this, Foxp3 expression is related with Treg differentiation and function. Studies have shown that forced expression of Foxp3 converts naïve T cells to

functionally suppressive Tregs (Fontenot et al., 2003). As shown in Fig 3.1f, the increased expression of Foxp3 on NO-induced CD25+Foxp3+CD127- cells suggested they may have higher immune regulatory capacity. Lastly, it is reported before that NO mediates UV-induced immune suppression by inhibiting DC function (Yuen et al., 2002). Consistent with these previous findings (Bogdan, 2001), NO significantly decreased HLA-DR and slightly decreased CD86 expression on immature mo-DC. Therefore, the possibility that NO also contributed to Foxp3+Treg induction indirectly could not be ruled out. Taken together, these data indicated that the enrichment of Foxp3+CD127- T cells by NO involved conversion of CD25lo/- cells and Treg proliferation.

Foxp3+ Tregs have been reported to utilize a number of mechanisms to exert their immunosuppressive function. Both *in vitro* and *in vivo* studies have proved that membrane-bound TGFβ was critical for nTreg function (Gandhi et al., 2010). Following a post-translational maturation, the amino terminal domain of TGFβ binded non-covalently with latency-associated peptide (LAP), which was a pro-peptide, forming an inactive form of TGF-β. Nakamura et al showed that mouse CD4+LAP+ Tregs exerted their suppressive function via a TGFβ–dependent manner and were effective in suppressing autoimmunity in a murine model of multiple sclerosis (Nakamura et al., 2004). Chen et al showed that responder cells up-regulated the expression of TGFβ receptor type II after being activated through TCR, which provided the opportunity for suppressor derived TGFβ to bind to activated responder cells and transduced suppressive signals via contact-dependent manner, as evidenced by the increased phosphorylation of Smad2/3 in responder cells upon contact with suppressor cells (Chen and Wahl, 2003). Therefore, the compound SB431542 was used to target the TGFβ signalling pathway. This compound acts as competitive ATP binding site kinase inhibitor leading to specific inhibition of kinase activity of TGFβ receptor type I and phosphorylation

of SMAD2 and SMAD3. Addition of SB431542 partially reversed the inhibition of proliferation caused by NO-induced CD25hi cells. The partially reversed inhibition indicated that membrane bound TGFβ is not the only mechanism utilized by NO-induced Foxp3+ Tregs. Besides membrane bound TGFβ, CTLA-4 and PD-1 are two key negative regulatory molecules that critically affect peripheral T-cell function. Active form of membrane bound TGFβ and TGFβ receptor type II expression on CD4+CD25+ suppressor T cells were up-regulated by CTLA-4 ligation, which suggested that it was possible that these pathways may work together to control inflammation. Here, I have shown that addition of antibodies blocking PD-1/PD-L1 also partially reversed the inhibition of proliferation caused by NO-CD25hi while blocking CTLA-4 did not have any effect, which was in consist with previous studies showing that NO-Tregs induced in mice did not require CTLA-4 but IL-10 for their suppressive function. Both membrane bound TGFβ and PD-1/PD-L1 pathways partially reversed the inhibitory effect of NO-induced Foxp3+ Tregs, however, neither of them played a dominant role. To further analyse whether there was addictive effect for these two pathways, NO-CD25hi cells were co-cultured with effector T cells in the presence of both TGFβ signalling inhibitor SB431542 and antibodies blocking PD-1/PD-L1. No synergistic effect was observed, which further confirmed that NO-induced Foxp3+ Treg use multiple pathways to exert its regulatory function to meet the challenges from distinct subsets of effector cells or in different tissues. The differential expression patterns of the CTLA- 4 and PD-1 led to the hypothesis that PD-1 controlled T cell function in tissue-specific sites due to its constitutive expression and CTLA-4 controls T cells in lymphoid tissue due to B7 expression on APC. Gene expression profiling of CD4+ T cell co-stimulated with PD-1 and CTLA-4 showed that PD-1 had stronger inhibitory effect than CTLA-4 on T cell activation (Francisco et al., 2010). Considering the enhanced expression of skin homing marker CLA on NO-induced Foxp3+Tregs and the involvement of PD-1/PD-L1 in mediating its suppressive function,

these results suggested that systemically increased NO generated by UV irradiation may also contribute to the control of cutaneous inflammation via Fopx3+Tregs

Summary

- NO induces Foxp3+Tregs with enhanced potential skin-migrating ability from human peripheral CD4+ T cells via c-GMP dependent ways.

- NO induced Fopx3+Tregs involves conversion of CD4+CD25- cells to CD25+Foxp3+CD127- cells and Fopx3+ T cells proliferation.

- The suppressive function of NO-induced Foxp3+Treg requires cell-cell contact and is mediated by multiple pathways including PD-1/PD-L1 and membrane bound TGF-β but not CTLA-4.

Chapter 4 Investigating the effect of *cis*-urocanic acid on the differentiation and function of human peripheral CD4+ T cells and monocyte-derived dendritic cells

4.1 Introduction

Urocanic acid (UCA), located in the stratum corneum, is a major epidermal chromophore. UCA is also suggested to play a role in UV-induced immunosuppression (Beissert and Schwarz, 2002). It is a breakdown product of filaggrin produced by histidase, and isomerises from trans-UCA to *cis*-UCA after UV radiation. *Cis*-UCA acts as a constitutive photo protectant that can reduce UV radiation induced deleterious effect by 33% (Gibbs and Norval, 2011). The isomerisation takes place at 300 – 315 nm in mouse skin and at 280 – 310 nm in human skin. UVA-II (320 – 340 nm) or UVA-I (340 – 400 nm) radiation can also induce the formation of *cis*-UCA % (Gibbs and Norval, 2011). It is reported that the action spectrum of UV induced suppression of contact hypersensitivity fits with the absorption spectrum of UCA but not of DNA or other components in the epidermis (Jones et al., 1996). The concentration of *cis*-UCA is elevated in UV-exposed skin in both human and mice. *Cis*-UCA has been reported to remain elevated in the serum of UV-irradiated mice. *Cis*-UCA concentration is increased in the circulation as well as in urine of UVB-irradiated individuals and this can last for at least two weeks. Systemically distributed *cis*-UCA upon photo-isomerization has been shown to induce systemic immune suppression (Gibbs et al., 2008). Removing the stratum corneum by tape stripping abrogates the UV induced suppression of contact hypersensitivity which suggests that UCA plays a role in UV-induced immunosuppression (Beissert and Schwarz, 2002).

Multiple cellular targets have been suggested to be involved in *cis*-UCA mediated immune suppression including neutrophils, keratinocytes, monocytes and T cells.

4.1.1 *Cis*-UCA mediated immune suppression in murine models

Several studies have reported that UV induced *cis*-UCA suppresses cellular immune responses including contact hypersensitivity (CH) and delayed type hypersensitivity (DTH) (Applegate et al., 1989). This is further evidenced by murine studies showing that depleting the *in vivo* function of *cis*-UCA using specific Ab inhibits suppression of DTH induced by UV radiation (Moodycliffe et al., 1996). Ab against *cis*-UCA also prevents UV-induced photocarcinogenesis (Beissert et al., 2001). *In vivo* experiments have shown that *cis*-UCA induces ROS leading to DNA damage, which is suggested to trigger inhibition of DTH (Sreevidya et al., 2010).

4.1.2 *Cis*-UCA mediated suppressive effect on CD4+ T cells and antigen presenting cells

Cis-UCA down-regulates the antigen-presenting ability of mouse spleen cells by inducing IL-10 secretion from activated CD4+ T cells (Moodycliffe et al., 1996). Langerhans cells ability to process and present antigen *in vitro* is significantly inhibited by *cis*-UCA, the effect of which can be reversed by addition of IL-12 (Beissert et al., 2001). The number of Langerhans cells is reduced by 62% after topical application of 20 μg of *cis*-UCA, which leads to significant suppression of mixed skin lymphocyte reaction (Norval et al., 1990).

4.1.3 *Cis*-UCA mediated improvement of skin inflammation

In murine models of DMSO induced-acute skin inflammation and 12-O-tetradecanoylphorbol-13-acetate (TPA) induced-subacute skin inflammation, 2.5% and 5% *cis*-UCA suppressed inflammation and attenuated skin oedema and erythema (Laihia et al., 2012). The clinical efficacy of *cis*-UCA in treating human skin conditions has also been investigated. 5% *cis*-UCA emulsion cream was well tolerated and effective in reducing trans-epidermal water loss and improving skin barrier function in both healthy volunteers and AD

patients; eczema area severity index was improved by 5% *cis*-UCA in mild-to-moderate AD (Peltonen et al., 2014). Topical application of *cis*-UCA prevented the induction of sensitization to DNCB on human skin (Dahl et al., 2010). A clear anti-psoriatic effect was observed when *cis*-UCA was used as topical treatment for psoriatic plaques (Vestey and Norval, 1997)

Hence, *cis*-UCA is a promising drug for AD but the molecular mechanisms involved in its action are still unclear. A number of studies have failed to demonstrate *cis*-UCA mediated immunosuppression *in vitro* including mitogen-induced human peripheral blood mononuclear cell (PBMC) proliferative responses, human mixed lymphocyte reaction (MLR) and antigen-specific T lymphocyte responses (Gibbs, Tye et al. 2008), implying that *cis*-UCA may have to interact with other cells in a more complex fashion than can be reproduced *in vitro* or that the suppression may be mediated by other factors induced by *cis*-UCA *in vivo*.

As understanding the mechanisms by which *cis*-UCA suppresses immune responses is important for AD treatments, this chapter describes experiments designed to determine the role of *cis*-UCA in modulating phenotype and function of human peripheral CD4+ Tcells and monocyte derived-DCs.

Chapter hypothesis and research questions

Cis-UCA contributes to the clinical efficacy of NB-UVB phototherapy in AD through its effects on the function of regulatory T cells either directly, or indirectly through its action on antigen presenting cells.

- Does *cis*-UCA induce functional regulatory T cells from human peripheral CD4+ T cells?
- Does *cis*-UCA modulate CD4+ T cell function directly or indirectly through antigen presenting cells?

4.2 Results

4.2.1 *cis*-UCA reduces CLA expression and CD25hiFoxp3+Treg induction from human peripheral CD4+ T cells.

I first investigated the effect of *cis*-UCA on CD4+ T cell phenotype and particularly on the induction of CD25hiFox3+ Tregs. In order to determine the optimal concentration of *cis*-UCA to use, human peripheral blood CD4+ T cells were purified and cultured with plate bound anti-CD3 and anti-CD28 antibodies in the presence of different concentrations of *cis*-UCA for 5 days as described in Chapter 2.3.1.2. The expression of CD25 and Foxp3 were analyzed by flow cytometry. 100 μg/ml *cis*-UCA has been used in murine *in vitro* and *in vivo* experiments (Beissert et al., 2001). Kanesaki reported that *cis*-UCA levels in human plasma, obtained after a total-body UV-B exposure of 250 J/m2 was approximately 69 μg/ml (Kanesaki et al., 1993). It is known that the skin concentration of UCA varies considerably between individules. Results showed that cis-UCA at 100 μg/ml had the strongest effect on induction of CD25hi Foxp3+ T cells and viability of CD4+ T cells was not affected (Fig 4.1 a & b). Thus, *cis*-UCA at 100 μg/ml was used for the following experiments.

CLA interacts with E-selectin expressed on endothelial cells and directs T cell migration to the skin. To investigate whether *cis*-UCA affects T cell migration, CLA expression was analyzed by flow cytometry. As shown in Fig 4.1c, *cis*-UCA significantly decreased the frequency of CLA+ T cells which suggested that fewer activated CD4+ T cell could migrate to skin following exposure.

Next *cis*-UCA's effect on Tregs was investigated. As CD127 staining was not used in this experiment, CD25hi Foxp3+ was used to gate Treg cells. As shown in Fig 4.1 d &e, the

percentage of CD25+ cells and geometric MFI of CD25 was not affected by *cis*-UCA indicating that T cell activation was not affected. As shown in Fig 4.1f, the percentage of CD25hi Fopx3+ cells was significantly decreased by *cis*-UCA as was CLA expression on CD25hi Foxp3+ cells.

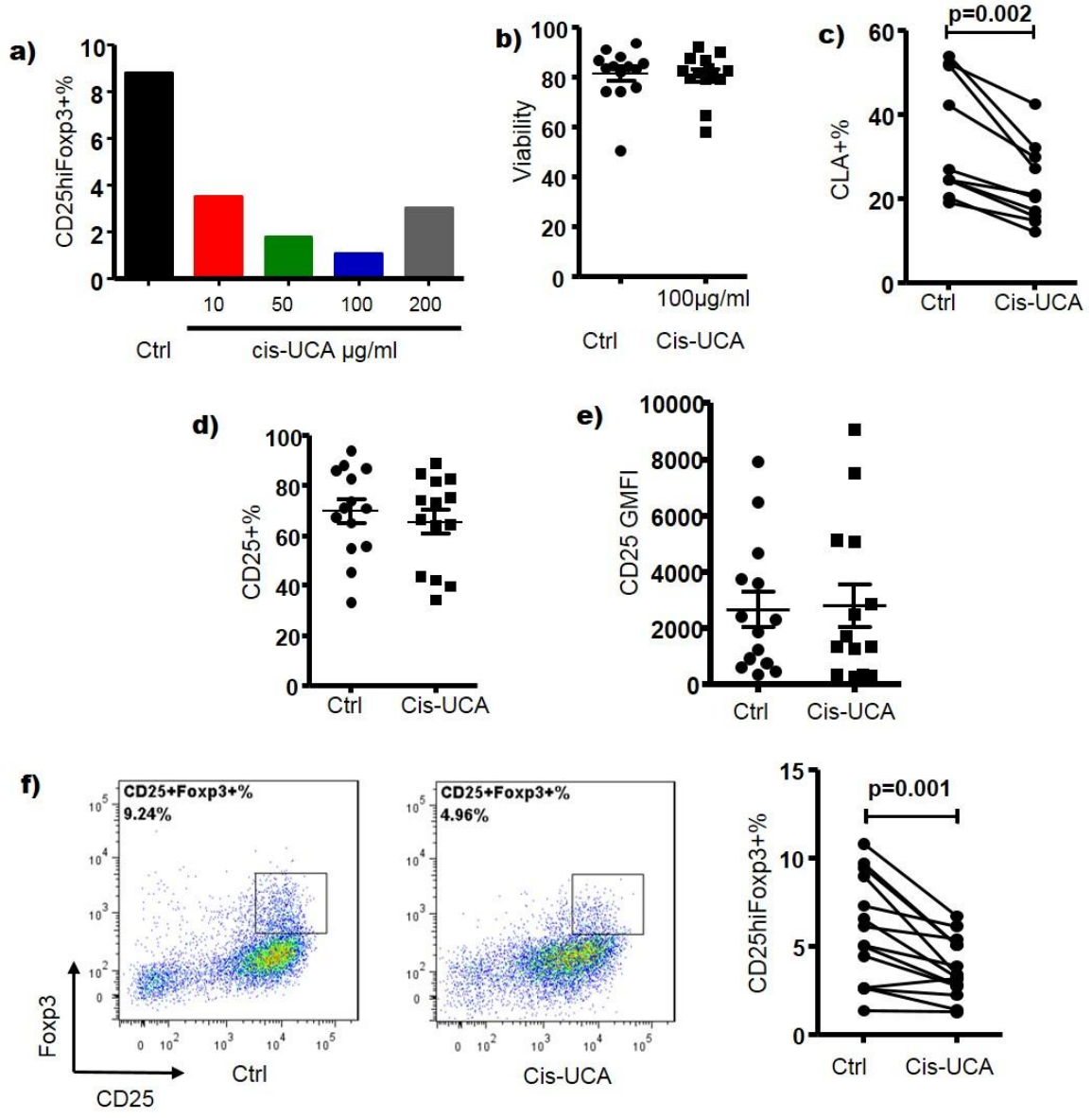


Fig 4.1 *cis*-UCA modulates human peripheral CD4+ T cell phenotype. Purified peripheral CD4+ T cells were activated with plate bound anti CD3/28 and treated with *cis*-UCA for 5 days. T cell phenotype was measured by flow cytometry. a) Percentage of CD25hi Foxp3+ cells of total CD4+ T cells after being

treated with different concentrations of *cis*-UCA. b) Viability of total cells after being treated with 100 µg/ml *cis*-UCA (N=14). c) Percentage of CLA+ cells of total CD4+ T cells after being treated with 100 µg/ml *cis*-UCA for 5 days (N=10). d-e) Percentage of CD25+ cells of total CD4+ T cells and geometric mean of CD25 after being treated with 100 µg/ml *cis*-UCA for 5 days (N=14). f) Representative FACS plot and the percentage of CD25hi Foxp3+ T cells after being treated with or without 100 µg/ml *cis*-UCA for 5 days (N=14). P value was calculated using Wilcoxon matched pairs T test. Ctrl = control cultures without *cis*-UCA

4.2.2 *cis*-UCA modulates CD4+ T cell phenotype through 5-HT2a receptor.

To date, little is known of how *cis*-UCA exerts its inhibitory effect. Systemic administration of serotonin receptor 2A (5-HT2aR) antagonist is reported to block *cis*-UCA induced immune suppression in a mouse model of DTH (Walterscheid et al., 2006). Therefore, I hypothesized that *cis*-UCA modulates CD4+ T cell phenotype through the 5-HT2a receptor.

To assess whether human CD4+ T cells express 5-HT2aR, protein was extracted from purified CD4+ T cells that were either unstimulated or stimulated with plate bound anti-CD3/28 for 5 days. The expression of 5-HT2aR was analyzed using western blot. As shown in Fig 4.2a, resting CD4+ T cells expressed a low level of 5-HT2aR and the expression was increased in activated CD4+ T cells.

To further investigate whether *cis*-UCA modulates CD4+ T cell phenotype through 5-HT2aR, an agonist for 5-HT2aR was used. Due to availability of the compounds, α-methylserotonin (αMS) which is an agonist against all three subtypes of 5-HT2 receptor was used. CD4+ T cells were activated with plate bound anti-CD3/28 in the presence or absence of 1nM αMS for 5 days and T cell phenotype was analyzed by flow cytometry. αMS had similar effects to

cis-UCA on CD4+ T cell phenotype, as evidenced by decreasing the percentage of CLA+ cells and CD25hi Foxp3+ cells. (Fig 4.2b).

Due to unspecificity of αMS, a specific antagonist of 5-HT2aR ketaserin (Ke) was used to further confirm whether 5-HT2aR mediates *cis*-UCA effect on CD4+ T cells. CD4+ T cells were activated with plate bound anti-CD3/28 and treated with 100μg/ml *cis*-UCA in the presence or absence of different concentrations of Ke for 5 days and T cell phenotype was analyzed by flow cytometry. As shown in Fig 4.2c, the modulatory effect of *cis*-UCA on the frequency of CD25hi Foxp3+ T cells was reversed by Ke. However, *cis*-UCA effect on the frequency of CLA+ T cells was not affected by the presence of Ke (Fig 4.2c), which indicated that 5-HT2aR may not be the only receptor for *cis*-UCA on CD4+ T cells.

Murine naïve CD4+ T cells have been reported to express tryptophan hydroxylase-1 (TPH-1) after activation which is the rate-limiting enzyme in serotonin synthesis (Leon-Ponte et al., 2007). To test whether purified human CD4+ T cells express TPH-1 leading to the production of endogenous serotonin, the expression of TPH-1 was analyzed using real-time PCR. As shown in Fig 4.3, there was no difference in TPH-1 expression between resting and activated CD4+ T cells and *cis*-UCA did not modulate TPH-1 expression indicating that *cis*-UCA did not modulate CD4+ T cell phenotype and function through endogenous serotonin.

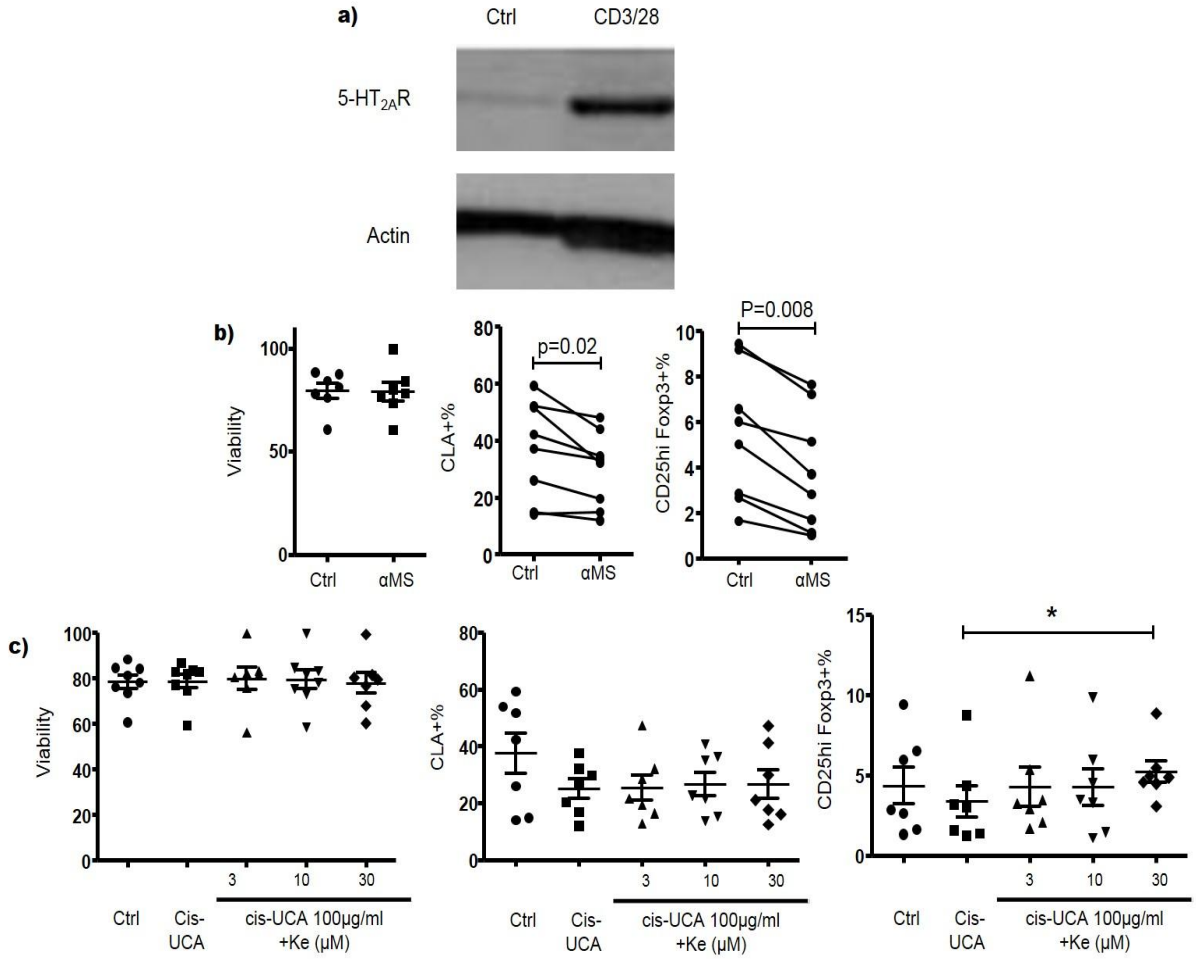


Fig 4.2 *cis*-UCA modulates CD4+ T cell phenotype through 5-HT2a receptor. Purified peripheral CD4+ T cells were activated with plate bound anti CD3/28 and treated with 1nM 5-HT2aR agonist α-methylserotonin (αMS) or 100 μg/ml *cis*-UCA in the presence of different concentrations of 5-HT2aR antagonist ketaserin (Ke) for 5 days. T cell phenotype was analyzed by flow cytometry. a) Western blot of 5-HT2aR expression on resting and activated CD4+ T cells. b) Viability, percentage of CLA+ cells and CD25hi Foxp3+ cells of total CD4+ T cells after being treated with 1nM αMS for 5 days (N=8). c) Viability, percentage of CLA+ cells and CD25hi Foxp3+ cells of total CD4+ T cells after being treated with *cis*-UCA in the presence of different concentrations of ketaserin for 5 days (N=7). P value was calculated by Friedman test followed by Dunn's post-test.

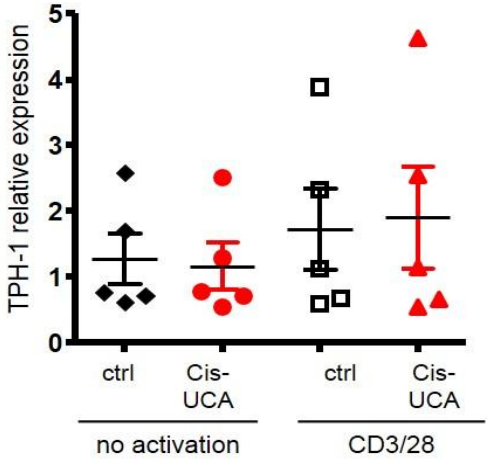


Fig 4.3 Human purified CD4+ T cell TPH-1 expression is not changed on activation. Purified peripheral CD4+ T cells were either left unstimulated or activated with plate bound anti CD3/28 for 3 days in the presence or absence of 100μg/ml *cis*-UCA. TPH-1 expression was analyzed using real-time PCR (N=5).

4.2.3 *cis*-UCA modulates CD4+ T cell function.

4.2.3.1 *Cis*-UCA affects CD4+ T cell cytokine secretion.

To further assess *cis*-UCA effect on CD4+ T cell cytokine secretion, a proteome profiler human cytokine array was performed. Cell culture supernatants from 4 donors were pooled together to minimize variations in individuals. Pooled culture supernatant from CD4+ T cells that had been activated with anti-CD3/28 for 5 days in the presence or absence of *cis*-UCA were analyzed. The result of the array is shown in Fig 4.4a. All cytokine/chemokines were represented in duplicate. Pixel intensity of the dots were analyzed using ImageJ software. Based on the semi-quantitative results, CCL4 (red boxes, Fig 4.4a) was further analyzed using ELISA. As shown in Fig 4.4b, *cis*-UCA significantly increased CCL4 secretion from activated CD4+ T cells.

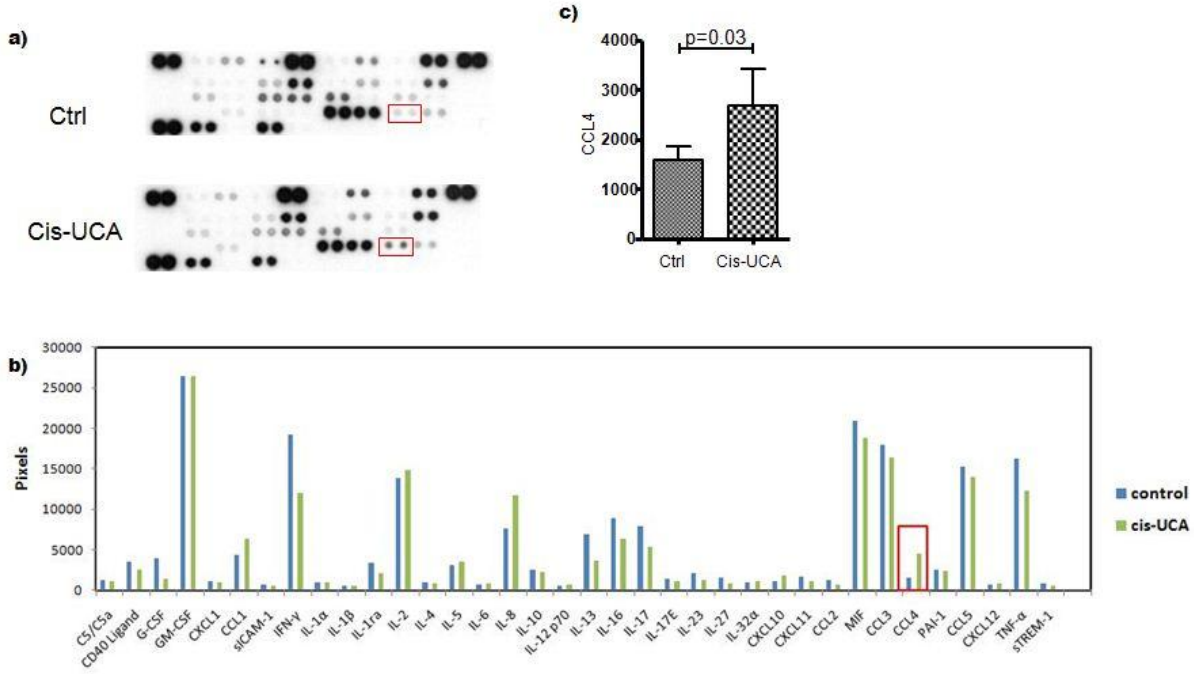


Fig 4.4 *Cis*-UCA affects CD4+ T cell cytokine secretion. CD4+ T cells were activated with plate bound anti CD3/28 and treated with 100 μg/ml *cis*-UCA. Cytokine secretion was analyzed by ELISA. a) Original figure of proteome profiler human cytokine array. Dots representative of CCL4 have been highlighted in red b) Densitometry analysis of original figure of proteome profiler human cytokine array. c) CCL4 concentration in the culture supernatant of activated CD4+ T cells being treated with or without 100 μg/ml *cis*-UCA for 5 days (N=7). P value was calculated using Wilcoxon matched pairs T test.

Previous report has shown that *cis*-UCA promoted the secretion of IL-10 from activated CD4+ T cells (Holán et al., 1998). As shown in Fig 4.5, *cis*-UCA significantly increased IL-10 secretion from activated human peripheral CD4+ T cells. Although IFNγ was slightly increased by *cis*-UCA as well, it did not reach significance. The IL-10/ IFNγ ratio was also significantly increased by *cis*-UCA.

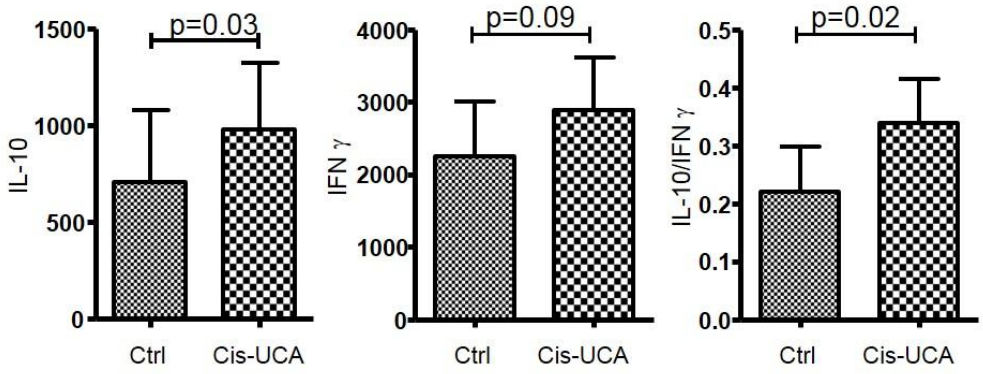


Fig 4.5 *cis*-UCA enhances the secretion of IL-10 from activated peripheral CD4+ T cells. Purified peripheral CD4+ T cells were activated with plate bound anti CD3/28 and treated with *cis*-UCA for 5 days. IL-10 (N=15) and IFNγ (N=17) concentration were analyzed using ELISA and the ratio of IL-10/ IFNγ (N=15) was calculated as well. P value was calculated using Wilcoxon matched pairs T test.

4.2.3.2 *Cis*-UCA inhibits CD4+ T cell proliferation.

Cis-UCA has been reported to inhibit the proliferation of rat bladder cancer cell line in a dose dependent manner (Arentsen et al., 2012). To investigate the effect of *cis*-UCA on CD4+ T cell proliferation, purified CD4+ T cells pre-labelled with the proliferation dye ef670 were activated with anti-CD3/28 and treated with 100 μg/ml *cis*-UCA for 4 days. Proliferation of CD4+ T cells was analyzed by flow cytometry. As shown in Fig 4.6, *cis*-UCA significantly decreased proliferation of activated CD4+ T cells which may contribute to its ability to control inflammation.

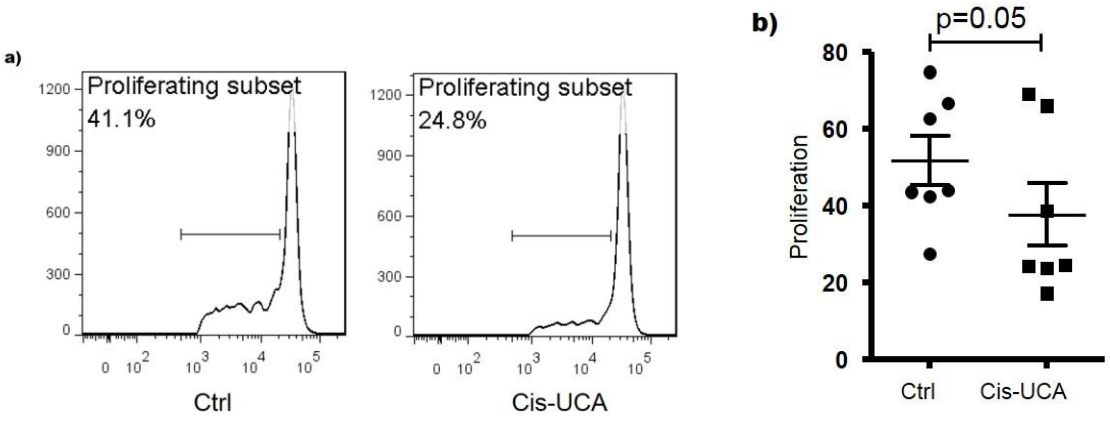


Fig 4.6 *cis*-UCA inhibits CD4+ T cell proliferation. Pre-labelled ef670 CD4+ T cells were activated with plate bound anti CD3/28 and treated with 100 μg/ml *cis*-UCA for 4 days. Proliferation of CD4+ T cells was measured by flow cytometry. a) Representative FACS plot of CD4+ T cell proliferation. b) CD4+ T cell proliferation after being treated with 100 μg/ml *cis*-UCA for 4 days (N=7). P value was calculated using Wilcoxon matched pairs T test.

4.2.3.3 *cis*-UCA modulates ERK signalling pathway in CD4+ T cells

The ability of *cis*-UCA to affect T cell function indicates that it can trigger signaling. *Cis*-UCA has been shown to trigger ERK signaling in T cell lines that are isolated from multiple sclerosis patients (Correale and Farez, 2013). Extracellular Signal-Regulated Kinases 1 and 2 (ERK) and p-38 are members of the Mitogen Activated Protein Kinase (MAPK) family. Therefore, we set out to determine the effect of *cis*-UCA on ERK signaling pathways. Purified CD4+ T cells were first starved by culturing them in RPMI without FBS at 37 degree for 2h before being activated with plate bound anti CD3/28 in the presence or absence of 100 μg/ml *cis*-UCA alone or with 5-HT2aR antagonist. The T cell pellets were collected at different time points and phosphorylation of ERK was analyzed by western blot.

As shown in Fig 4.7a, T cell activation led to increased phosphorylation of ERK, as expected. Addition of *cis*-UCA however decreased ERK phosphorylation (p-ERK) which was further

confirmed by densitometry analysis shown in Fig 4.7b. *Cis*-UCA's effect on ERK phosphorylation was reversed by 5-HT2a antagonist ketaserin (Fig 4.7 a&b) (N=3).

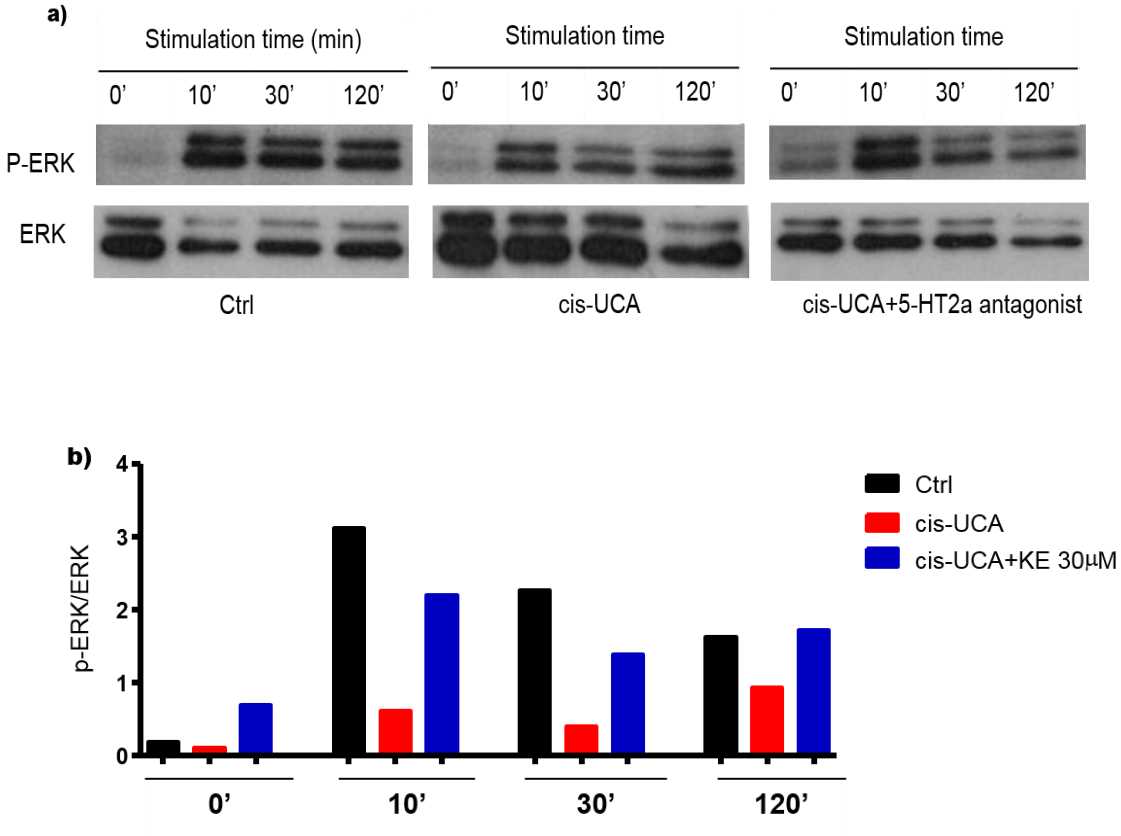


Fig 4.7 *cis*-UCA modulates ERK signalling pathway in activated CD4+ T cells. Purified CD4+ T cells were starved for 2h before being activated with plate bound anti CD3/28 in the presence or absence of 100 μg/ml *cis*-UCA alone or with 5-HT2aR antagonist. The plate was spun down and T cell pellets were collected at different time points and proteins were extracted using RIPA buffer. Phosphorylation of ERK was analyzed by western blot. a) Representative western blot image of *cis*-UCA and 5-HT2aR antagonist ketaserin effect on ERK phosphorylation. b) Densitometry analysis of *cis*-UCA and 5-HT2a antagonist ketaserin effect on ERK phosphorylation. Representative result of 3 separate experiments.

4.2.3.4 *cis*-UCA modulates vitamin D effect on CD4+ T cells by decreasing vitamin D receptor (VDR) expression.

Besides *cis*-UCA, $1,25(OH)_2D_3$ (vitD) is an important mediator derived from UV radiation

with immune regulatory functions which has direct effects on CD4+ T cells and promotes differentiation and suppressive function of Foxp3+Tregs. C*is*-UCA has the opposite effect of vitD on CD25hi Foxp3+ T cells differentiation. To analyze whether *cis*-UCA can affect the effect of vitD on Foxp3+Tregs induction, purified human peripheral CD4+ T cells were activated with plate bound anti-CD3/28 and treated with either 100 μg/ml *cis*-UCA or 10^{-7}M vit D or both for 5 days. As shown in Fig 4.8a, vit D alone enhanced the frequency of CD25hi Foxp3+ T cells as reported before, and addition of 100 μg/ml *cis*-UCA partially reversed this effect. Taken together, these results indicated that a fine balance may exist between UV-induced anti-inflammatory molecules in terms of their ability to suppress immune responses.

The biological effects of vit D are mediated by the vitamin D receptor (VDR). ERK signaling is suggested to be involved in VDR expression (Gocek et al., 2007). Since we have observed that *cis*-UCA partially reversed the effect of vit D on CD25hiFoxp3+ cell induction, we further investigated whether *cis*-UCA modulates vit D's effect by controlling its receptor. Purified CD4+ T cells were stimulated with plate bound anti-CD3/CD28 and treated with either 100 μg/ml *cis*-UCA or 10^{-7} M vit D or both for 3 days. The expression of VDR was analyzed using real-time PCR. VDR expression was increased after activation and vit D treatment further increased expression of VDR. However, upon stimulation, VDR expression in CD4+ T cells that have been treated with *cis*-UCA was decreased. *Cis*-UCA also partially reversed the effect of vit D on VDR expression as shown in Fig 4.8b, which suggested that *cis*-UCA may modulate biological effect of vit D via decreasing expression of VDR.

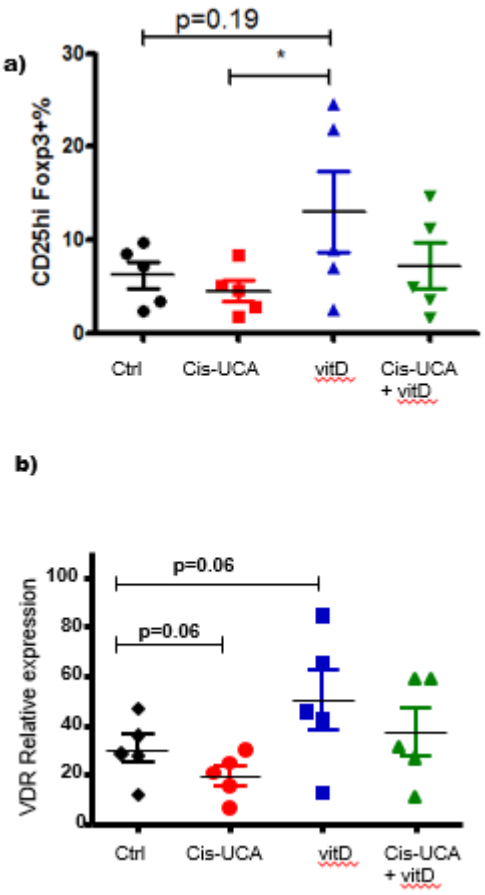


Fig 4.8 *cis*-UCA modulates vitamin D effect on CD4+ T cells by decreasing VDR expression. Purified peripheral CD4+ T cells were activated with plate bound anti CD3/28 and treated with 100 µg/ml *cis*-UCA or 10^{-7} M vit D or both for 5 days for phenotype analysis or 3 days for VDR expression analysis. T cell phenotype was analyzed by flow cytometry and VDR expression was measured by real-time PCR. a) Percentage of CD25hi Foxp3+ cells of total cells after being treated with *cis*-UCA or vit D or both for 5 days (N=5). b) VDR expression of total cells after being treated with *cis*-UCA or vit D or both for 3 days (N=5). 18s was used as house keeping gene. P value was calculated by Friedman test followed by Dunn's post-test.

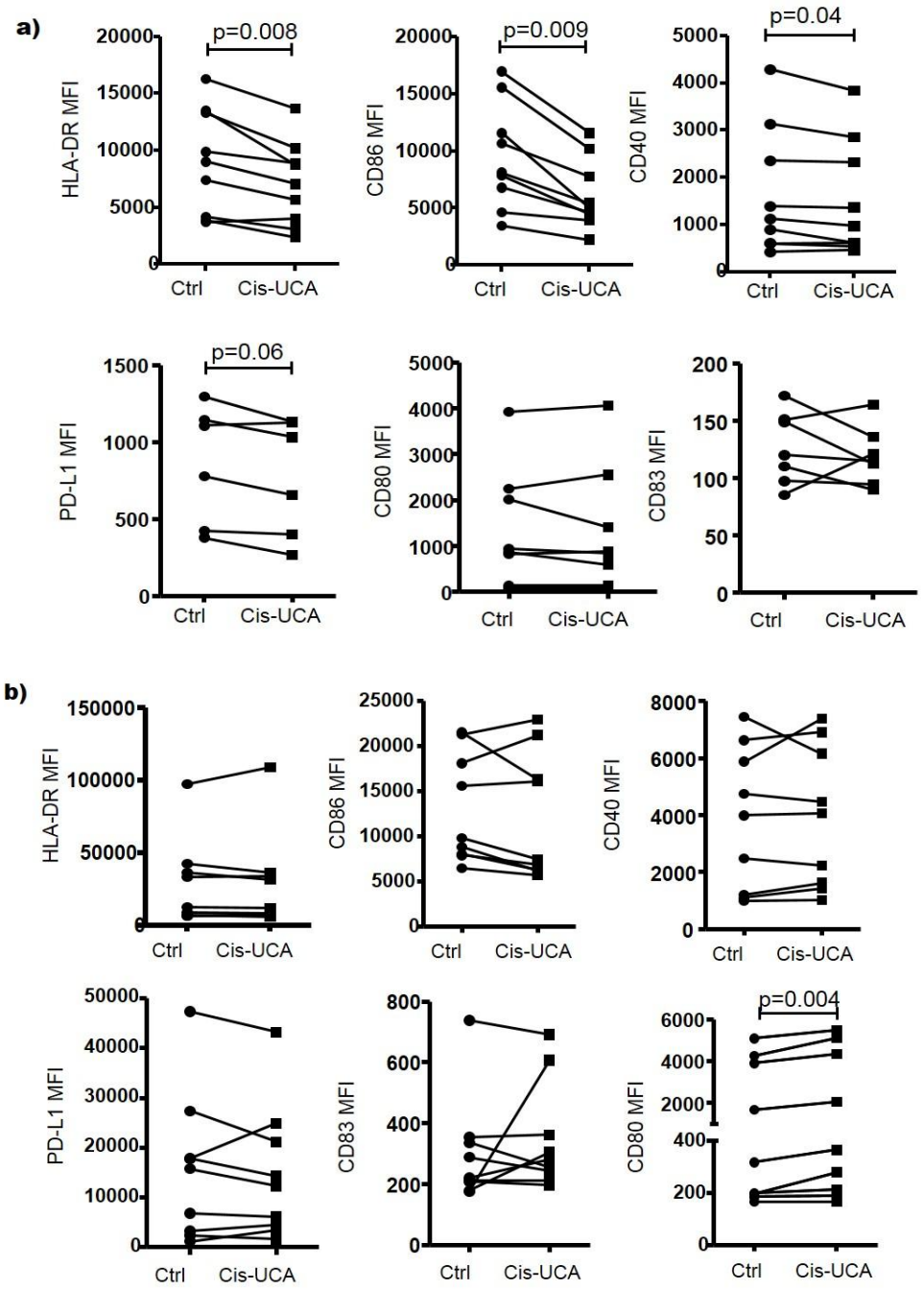
4.2.4 *cis*-UCA modulates mo-DCs phenotype.

Naïve T cells activated by antigen presenting cells differentiate into effector T cells or

regulatory T cells which suggests that antigen presenting cells play an important role in the development of an inflammatory response. In AD patients, skin barrier defects, increased exposure of epidermal DCs to irritants, microbial danger signals and antigens which penetrate skin can lead to the immunological changes of atopic eczema (Kubo et al., 2012). *Cis*-UCA's effect on antigen presenting cells remains elusive. To address this issue, we analyzed the effect of *cis*-UCA on human monocyte-derived DCs (mo-DCs). CD14+ monocyte were positively selected from PBMCs and differentiated with human GM-CSF, IL-4 for 6 days before being matured with LPS or PolyI/C for 24 hours or 48 hours, respectively. Phenotype of both immature and matured mo-DCs was analyzed by flow cytometry.

As shown in Fig 4.9a, *cis*-UCA significantly decreased the expression of co-stimulatory molecules CD86 and CD40 and antigen presenting molecule HLA-DR on immature mo-DCs. There was a slight decrease of PD-L1 expression caused by *cis*-UCA on immature mo-DCs although it did not reach significance. The expression of co-stimulatory molecule CD80 and the maturation marker CD83 was not affected by *cis*-UCA on immature mo-DCs.

In contrast, for mo-DCs matured with Poly I/C for 48h, *cis*-UCA did not have an effect on the expression of HLA-DR, CD86, CD40 and PD-L1 as shown in Fig 4.7b. *Cis*-UCA only significantly increased the expression of CD80 (Fig 4.9b). For mo-DCs matured with LPS, among all the surface markers measured, only the expression of PD-L1 was significantly decreased by *cis*-UCA which suggested that *cis*-UCA's effect on matured mo-DCs may be varied depending on stimulation (Fig 4.9c)


a)
HLA-DR MFI
p=0.008
CD86 MFI
p=0.009
CD40 MFI
p=0.04
PD-L1 MFI
p=0.06
CD80 MFI
CD83 MFI
Ctrl
Cis-UCA
b)
HLA-DR MFI
CD86 MFI
CD40 MFI
PD-L1 MFI
CD83 MFI
CD80 MFI
p=0.004
Ctrl
Cis-UCA

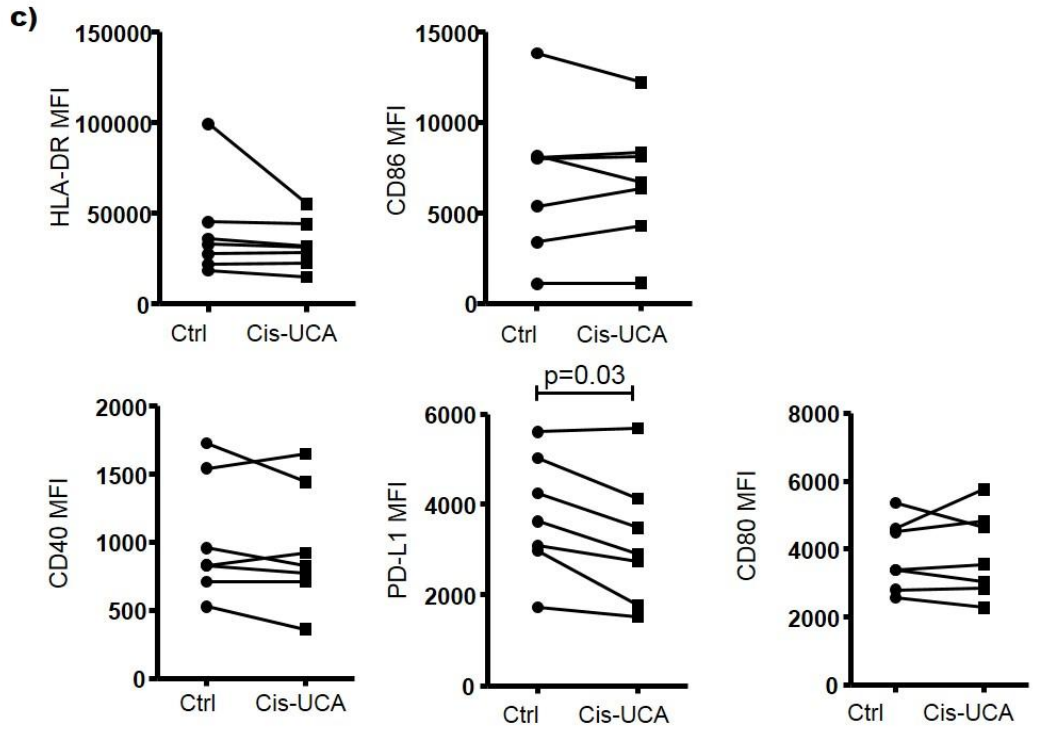


Fig 4.9 *cis*-UCA modulates human mo-DCs phenotype. CD14+ monocytes were isolated from PBMCs and cultured with cytokines including GM-CSF and IL-4 for 6 days with or without 100 μg/ml *cis*-UCA to generate immature mo-DCs. The immature mo-DCs were matured with 10ng/ml LPS or 20 μg/ml Poly I/C for 24h or 48 h, respectively. Phenotypes of both immature and mature mo-DCs were analyzed by flow cytometry. a) Expression of antigen-presenting molecule, costimulatory molecules and maturation molecules on immature mo-DCs. b) Expression of antigen-presenting molecule, costimulatory molecules and maturation molecules on mo-DCs matured with 20 μg/ml Poly I/C for 48h. c) Expression of antigen-presenting molecule, costimulatory molecules and maturation molecules on mo-DCs matured with 10ng/ml LPS for 24h. P value was calculated using Wilcoxon matched pairs T test.

4.2.5 *cis*-UCA promotes CD25+Foxp3+CD127- T cells induction indirectly by modulating mo-DCs.

The results above showed that in response to 100 μM *cis*-UCA, the expression of co-

stimulatory molecules and antigen presenting molecule were decreased in immature mo-DCs. In order to investigate whether these immature mo-DCs were indeed less immune stimulatory, co-culture experiments with allogenic CD4+ T cells were performed. CD14+ monocytes were positively selected from PBMCs and differentiated with human GM-CSF and IL-4 for 6 days to generate immature mo-DCs which were matured with 10ng/ml LPS for 24h. Allogenic CD4+ T cells were stained with proliferation dye ef670 before being co-cultured with immature or matured mo-DCs. Proliferation and phenotype of allogenic CD4+ T cell were analyzed by flow cytometry.

For allogenic CD4+ T cells that were co-cultured with immature mo-DCs differentiated in the presence or absence of *cis*-UCA, there was no difference in T cell proliferation (Fig 4.10 a). However, the percentage of CD25+Fopx3+CD127- cells was increased by co-culturing with immature mo-DCs differentiated in the presence of *cis*-UCA (Fig 4.10 b, N=3). The suppressive function of the induced CD25+Fopx3+CD127- cells was not tested in this project. To test this, further studies could be conducted to analyse the function of these cell by isolating the induced CD25+CD127- cells and co-culturing them with autologous effector T cells.

For allogenic CD4+ T cells that were co-cultured with mature mo-DCs differentiated in the presence or absence of *cis*-UCA, there were no changes in both parameters that were measured including T cell proliferation and percentage of CD25+Fopx3+CD127- cells (Fig 4.10 a&b, N=3).

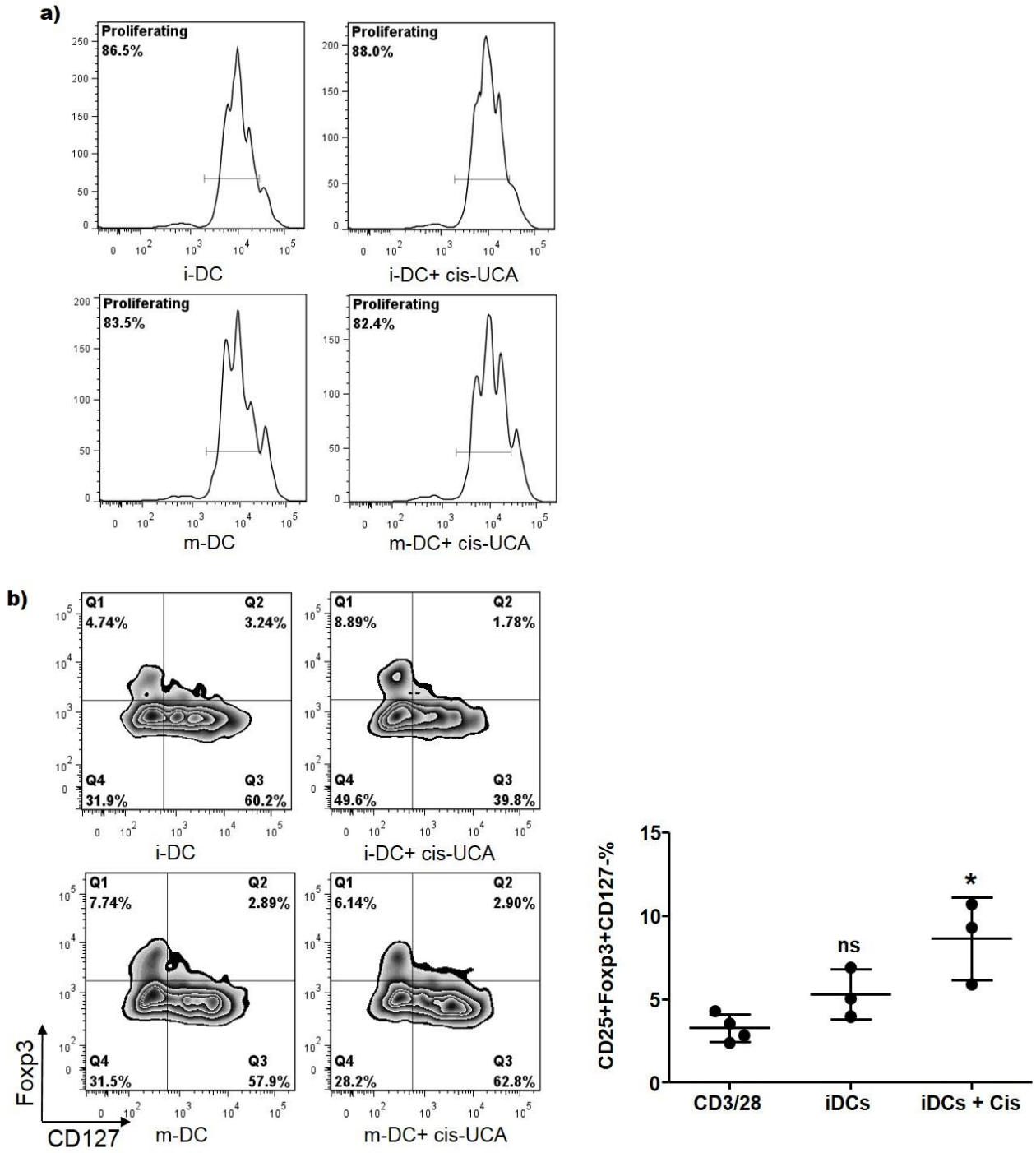


Fig 4.10 *cis*-UCA promotes CD25+Foxp3+CD127-T cells induction indirectly by modulating mo-DCs.

CD14+ monocyte were positively selected from PBMCs and differentiated with human GM-CSF, IL-4 for 6 days to generate immature mo-DCs (i-DC) in the presence or absence of 100 μg/ml *cis*-UCA. i-DC were matured with 10ng/ml LPS for 24h to generate matured mo-DCs (m-DC) in the presence or absence of

100 µg/ml *cis*-UCA. Allogenic CD4+ T cells were stained with proliferation dye ef670 before being co-cultured with immature or matured mo-DCs for 5 days. Proliferation and phenotype of allogenic CD4+ T cell were analyzed by flow cytometry. Representative FACS plot showing a) Proliferation b)) percentage of CD25+Foxp3+CD127- cells of allogenic CD4+ T cells after being co-cultured with immature or mature mo-DCs that have been generated with or without *cis*-UCA (Gated on CD25+ cells, N=3). CD4+ T cells activated with plate bound anti CD3/28 was used as positive control and CD4+ T cells that were left unstimulated was used as negative control. Kruskall Wallis test with Dunn's multiple comparison post-test, ns= not significant, * = $p<0.05$

4.3 Discussion

UVB radiation is absorbed by DNA, trans-UCA and cell membranes when it reaches the epidermis and upper dermis. Vitamin D production starts in skin following UV interactions with 7-dehydrocholesterol, which has long been thought to exert immunosuppression locally in skin and systemically (Schwarz et al., 2012). However, vitamin D is not the only anti-inflammatory molecule induced by UV irradiation and mediators other than vitamin D that are induced may also be involved in UV-mediated immunomodulation (Norval, 2006). UVB radiation causes photo-isomerization of trans-UCA to *cis*-UCA. *Cis*-UCA is reported to downregulate immune responses both locally and systemically. The local immuno-regulatory effect of *cis*-UCA is shown by the observation of Decara et al that the amount of *cis*-UCA detected in squamous cell carcinoma biopsy is 44% of total UCA which is significantly higher than that of healthy photo-exposed skin (Decara et al., 2008). The systemic immuno-modulatory effect of *cis*-UCA was investigated in a murine colitis model. *Cis*-UCA administered subcutaneously prevented weight loss and attenuated changes in colon weight/length in dextran sodium sulfate induced murine colitis model (Albert et al., 2010). Therefore, the modulatory effect of *cis*-UCA on human peripheral CD4+ T cell and mo-DC phenotype and function was investigated.

CLA interacts with E-selectin to mediate homing of CD4+ T cells to skin, which is considered as a biomarker for T cell mediated skin disease. CLA, as an inducible carbohydrate epitope of P-selectin glycoprotein ligand-1, is selectively expressed on activated T cells. Evidence suggests that systemic low dose UVB inhibited the migration of CD4+ T cells to skin by 50% during elicitation in a murine CHS model, which implies this might be one of the mechanisms by which UVB modulates elicitation of CHS (Rana et al., 2008). Thus, the decreased prevalence of CLA+ cells induced by *cis*-UCA in activated CD4+ T cells shown in Fig 4.1c

indicates that fewer CD4+ T cell would migrate to skin after UV radiation, which may contribute to the clinical observation that fewer infiltrating inflammatory cells are seen after UVB phototherapy.

The immunoregulatory function of vitD is well recognized including its effect on Tregs. A previous study has shown that *cis*-UCA promotes CD25+Foxp3+Treg induction from human PBMCs (Correale and Farez, 2013). Therefore, I investigated the percentage of CD25hi Foxp3+ T cell after different treatments in purified CD4+ T cells. Vitamin D significantly increased the prevalence of CD25hi Foxp3+ T cells, which is consistent with previous studies. In contrast to previous study, a significant decrease of proportion of CD25hi Foxp3+ T cell was observed after *cis*-UCA treatment. The difference between whole PBMCs that were used in the previous study and purified CD4+ T cells used here may contribute to the different results we have seen since *cis*-UCA may affect T cell phenotype and functions indirectly via APCs which will be shown later.

It is important to characterize the receptor(s) for *cis*-UCA which may provide further insights into the biological functions of *cis*-UCA, however, the receptor(s) for *cis*-UCA remains elusive. Using computational docking method, similar binding modes were observed for *cis*-UA and 5-HT with 5-HT2a receptor while binding affinity for *cis*-UCA was relatively higher than 5-HT (Shen and Ji, 2009). 5-HT2aR antagonist is also reported to block *cis*-UCA induced ROS *in vivo* (Sreevidya et al., 2010). All these evidence suggest *cis*-UCA mediates its biological function through 5-HT2aR. In contrast, several studies suggest that 5-HT2aR is not the receptor for *cis*-UCA. For example, *cis*-UCA induces secretion of IL-6, TNFα and PGE2 from keratinocytes, which cannot be blocked by 5-HT2aR antagonist (Kaneko et al., 2009). *Cis*-UCA effect on TNFα and PGE2 secretion from PBMC cannot be mimicked by 5-

HT2aR agonist DOI, which suggests *cis*-UCA effect is mediated by receptors other than 5-HT2aR (Woodward et al., 2006).

Here we have shown that resting purified CD4+ T cells expressed low levels of 5-HT2aR and that the expression of 5-HT2aR was increased after CD4+ T cells were activated by CD3/28, which indicated it may be the receptor for *cis*-UCA on CD4+ T cells. *Cis*-UCA effect on CD4+ T cell phenotype could be mimicked by 5-HT2aR agonist αMS. Due to the low specificity of αMS, the modulatory effect of a more specific 5-HT2aR antagonist ketaserin on *cis*-UCA effect on CD4+ T cell phenotype was investigated. *Cis*-UCA effect on prevalence of CD25hi Foxp3+ cells could be blocked by ketaserin in a dose dependent manner, however, its effect on prevalence of CLA+ cells could not. These results indicate that 5-HT2a at least partially mediates the biological effect of *cis*-UCA while other receptors might also be involved.

Many mechanisms through which *cis*-UCA might exercise its regulatory function have been proposed. *Cis*-UCA effect on cytokine secretion is suggested to be involved in its immunosuppressive function. Based on the results of cytokine array, *cis*-UCA effect on CCL4 secretion was analyzed. *Cis*-UCA significantly increased the secretion of CCL4 from activated CD4+ T cells, which was suggested to be immunosuppressive. B lymphocytes activated by B cell receptor recruit CD4+CD25+ Tregs which inhibit the production of T cell mediated self-reactive antibodies by secreting CCL4 (Bystry et al., 2001). CCR5, which is the receptor for CCL4, is expressed on murine CD4+CD25+T cells and only on a small subpopulation of CD4+CD25- T cells (Wysocki et al., 2005). Previous study has shown that *cis*-UCA induces IL-10 secretion from murine naïve CD4+ T cells (Holán et al., 1998). Here, I have shown that an increased secretion of IL-10 from activated CD4+ T cells was induced

by *cis*-UCA. This result indicated that IL-10 secreting type 1 regulatory T cells may be induced by *cis*-UCA. Although there are studies showing that CD49b and the lymphocyte activation gene-3 are indispensable for Tr1, as mentioned in Chapter 1.2.1.2.2, it is difficult to define Tr1 using surface markers (Gagliani et al., 2013). Therefore, further experiments should be done to define the source of IL-10. Although the secretion of pro-inflammatory cytokine IFNγ was slightly increased by *cis*-UCA as well, the effect of *cis*-UCA on IL-10 secretion was stronger than that on IFNγ leading to an increased IL-10/ IFNγ ratio. IL-10 plays an important role in UV induced immunosuppression as evidenced by the loss of UV-induced tolerance and transfer of suppression when IL-10 is neutralized by anti-IL-10 Abs. This result indicates that IL-10 may contribute to the systemic immunosuppressive function of *cis*-UCA. This notion is consistent with two previous studies. *In vivo* experiment shows that neutralizing *cis*-UCA using anti-*cis*-UCA mAb before UV irradiation blocks the production of IL-10 in spleen (Moodycliffe et al., 1996). *Cis*-UCA confers systemic immunosuppression in a murine intestinal inflammation model, the effect of which is lost in IL-10-/- mouse (Albert et al., 2010).

Besides cytokine secretion, *cis*-UCA's effect on ERK signaling has also been investigated. Studies have shown that ectopic activation of ERK in T cells promotes Treg generation (Li and Rudensky et al., 2016). Therefore, the decreased phosphorylation of ERK is in consist with the decreased CD25hi Foxp3+% caused by *cis*-UCA. ERK signaling has been suggested to promote polarization of T cells toward Th2 by modulating IL-4 receptor function both in vivo and in vitro (Yamashita et al., 1999). *Cis*-UCA was shown here to decrease phosphorylation of ERK, which may contribute to inhibition of Th2 differentiation.

Cis-UCA as an endogenous metabolite of histidine is reported to transport protons into the cytosol of cells from acidic extracellular medium. The intracellular acidification induced by

cis-UCA leads to decreased proliferation of several human cancer cell lines including human melanoma, cervical carcinoma, human bladder carcinoma and fibrosarcoma cells (Peuhu et al., 2010). In a murine xenograft model of human melanoma, intratumoral injection of *cis*-UCA leads to decreased proliferation of melanoma cells and increased tumor necrosis and caspase-3 activation (Peuhu et al., 2010). Consistent with previous studies, I have shown here that proliferation of purified human peripheral CD4+ T cells was significantly inhibited by *cis*-UCA. ERK signaling has been reported to promote T cell proliferation indirectly by modulating AP-1 activity (Chang and Karin, 2001). Therefore, the decreased phosphorylation of ERK signaling caused by *cis*-UCA that showed above may contribute to this effect. Taken together, these results indicate that *cis*-UCA may control inflammation by inhibiting T cell proliferation.

ERK signaling is suggested to be involved in VDR expression in activated CD4+ T cells (Gocek et al., 2007). Based on the decreased phosphorylation of ERK and decreased proportion of CD25hi Foxp3+ cells in activated CD4+ T cells induced by *cis*-UCA showed above, I also tested *cis*-UCA effect on VDR expression. Results showed that *cis*-UCA decreased the expression of VDR in activated CD4+ T cells after three days treatment. Furthermore, vitamin D's effect on CD25hi Fopx3+ cells and VDR expression were partially affected by addition of *cis*-UCA as shown in Fig 4.8. These results suggest that *cis*-UCA may modulate the effect of vitamin D on CD4+ T cells by affecting VDR expression.

Evidence suggests that the state of DC maturation could affect the development and function of regulatory T cells and promote T cell tolerance (Maloy and Powrie, 2001). Intravenous administration of *cis*-UCA has been shown to decrease antigen presenting ability of splenic DCs (Noonan et al., 1988). UVB irradiation decreases antigen presenting ability of spleen

cells, the effect of which is restored by systemic administration of anti-*cis*-UCA mAb (Moodycliffe et al., 1996). Hence, we investigated the effect of *cis*-UCA on mo-DCs phenotype and function. LPS was used to activate mo-DCs not only because it is widely used compound for innate immune activation but also because it is an important component of house dust mite (HDM) which is a predominant provider of aeroallergen and a risk factor for AD. Besides LPS, Poly I/C was also used as another stimulus to mature mo-DCs. LPS interacts with Toll like receptor (TLR) 2 and 4 while Poly I/C interacts with TLR 3, which simulate viral infection. *Cis*-UCA decreased the expression of antigen presenting molecule HLA-DR and co-stimulatory molecules CD86 and CD40 on immature DCs while the expression of these molecules was not affected in matured mo-DCs, which indicates that *cis*-UCA may inhibit immature mo-DCs differentiation. These results are different from previous study which showed that *cis*-UCA at 100 μg/ml decreased the expression of CD86 on LPS-matured mo-DCs (Leitch et al., 2016). As shown in Fig 4.9c, the expression of HLA-DR varied considerably among individules, which may contribute to the differences that showed here.To investigate antigen presenting function of *cis*-UCA treated mo-DCs, allogenic CD4+ T cells were co-cultured with either immature or mature mo-DCs. Early study has shown that *in vitro* treatment of splenic DC with *cis*-UCA does not affect its ability to stimulate proliferation of antigen-specific T cells (Moodycliffe et al., 1996). In the rat Trichinella spiralis infection model, mixed lymphocyte reaction is not modulated by subcutaneous injection of *cis*-UCA (LOVEREN, 1999). In keeping with this, there was no difference in T cell proliferation stimulated by either immature or mature mo-DCs cultured with or without *cis*-UCA as shown in Figure 4.10a. These results suggest that *cis*-UCA does not affect antigen presentation of immature and mature mo-DCs. Early studies have shown that spleen suppressor cells from UV irradiated mouse transferred to recipient mouse suppress delayed type hypersensitivity and contact hypersensitivity. This effect is blocked by anti-*cis*-UCA

mAb, which indicates the involvement of *cis*-UCA in the generation of spleen suppressor cells in UV irradiated mice (Moodycliffe et al., 1996). Interestingly, the percentage of CD25+Foxp3+CD127- cells in allogenic CD4+ T cells was increased by co-culture with immature mo-DCs differentiated in the presence of *cis*-UCA while mature mo-DCs cultured with *cis*-UCA did not have effect on this cell population. Therefore, these data suggest that *cis*-UCA indirectly modulates Foxp3+Treg induction through antigen presenting cells.

Taken together, these data suggest that *cis*-UCA is likely to be involved in multifaceted pathways reflecting its complex nature as an immunosuppressive molecule induced by UV irradiation. *cis*-UCA may exert its immuno-regulatory function by inducing production of immunosuppressive cytokines including IL-10 and CCL4, inhibiting phosphorylation of ERK, inhibiting CD4+ T cell proliferation and inhibiting the expression of co-stimulatory molecules on immature mo-DCs leading to generation of T cells with a T regulatory phenotype while it interferes with the immuno-regulatory function of vitamin D by inhibiting VDR expression.

Summary

- *Cis*-UCA may prevent excess activated T cells infiltrating the skin by decreasing the prevalence of CLA+ cells.
- 1,25(OH)2D and *cis*-UCA differentially affect CD25hi Foxp3+cell induction and the ratio of 1,25 (OH)2D and *cis*-UCA may determine the outcome of activated CD4+ T cells
- *Cis*-UCA modulates CD4+ T cell phenotype via 5-HT2aR.
- *Cis*-UCA modulates inflammatory responses by inhibiting CD4+ T cell proliferation, phosphorylation of ERK signaling and inducing the production of immunosuppressive cytokines including IL-10 and CCL4.
- *Cis*-UCA modulates 1,25 $(OH)_2$D effect on CD4+ T cell through VDR.
- *Cis*-UCA promotes CD25hi Foxp3+ cell induction indirectly via modulating mo-DCs

Chapter 5 Investigating the role of FLG genotype and biomarkers reflecting immune polarization in clinical efficacy of NB-UVB phototherapy

5.1 Introduction

Within the last two decades, NB-UVB phototherapy with wavelengths of 311 nm of UV irradiation has turned out to be a major therapeutic strategy for a variety of skin diseases, including psoriasis, mycosis fungoides, polymorphic light eruption and AD. In comparison with psoriasis, which is still the most frequent indication for NB-UVB phototherapy, a reasonable number of AD patients do not respond to the treatment or even get worse after phototherapy. In general, AD patients respond less reliably to phototherapy. The majority of studies investigating NB-UVB phototherapy focus on clinical responses while few of them have evaluated the mechanisms of NB-UVB phototherapy in patients with AD. There are only limited data on the molecular effects of UV radiation in patients with AD. The lack of mechanistic studies in therapeutic trials in patients with AD is partially due to the incomplete understanding of the pathogenic mechanisms contributing to the disease (Tintle et al., 2011).

It is thus important to identify characteristics of AD patients that will determine whether they will respond to phototherapy. Hence, characteristics such as genotype, biomarkers reflecting immune polarization and clinical phenotype could be used in clinical study design and drug development to select existing or novel therapies to patients most likely to benefit from a mechanism-based treatment.

It is well known that UV irradiation induces production of vitamin D which is suggested to be responsible for UV induced immunosuppression that contributes to clinical effect of NB-UVB phototherapy. For example, *in vitro* experiments have shown that vitamin D promotes

the production of anti-microbial peptide β-defensin 1 and 2 in keratinocytes which contributes to the enhanced expression of these anti-microbial peptides in patients with AD treated with NB-UVB phototherapy (Gambichler et al., 2006).

However, UV radiation can suppress contact hypersensitivity responses and allergic airway disease in vitamin D3-deficient mice indicating the immune functions may be controlled by vitamin D3-independent mechanisms (Gorman et al., 2012). Further studies have shown that UV radiation induces a similar extent of immunosuppression in mice which do not express vitamin D receptor (VDR) (Schwarz et al., 2012). Other studies showed that changes in vitamin D produced in skin by UV irradiation have not been correlated with local immune regulation (Hart et al., 2011)

Vitamin D is not the only anti-inflammatory molecule induced by UV irradiation. For example, UVR-induced prostaglandin E2 (PGE2) mediates systemic immunosuppression by reducing the ability of progenitor dendritic cells from the bone marrow to develop into effective antigen-presenting cells (Ng et al., 2010). Therefore, UV radiation induced mediators other than vitamin D may also be involved in immunomodulation (Norval, 2006).

As introduced in Chapter 1.4.1.4.2, NO is released from human skin upon exposure to UV irradiation. NO released by this process has been shown to have systemic modulatory effect. For example, Liu et al show that UVA irradiation induces NO production from human skin which leads to vasodilation of arterial vasculature and decrease of blood pressure (Liu et al., 2014). *cis*-UCA formation is also induced by UV irradiation (see Chapter 1.4.1.4.3). *Cis*-UCA may also have systemic effects as evidenced by systemic administration of an antagonist against the *cis*-UCA receptor which blocked *cis*-UCA induced immune suppression

(Walterscheid et al., 2006). *cis*-UCA is a breakdown product of filaggrin, therefore, FLG genotype can be used as an indication of cutaneous *cis*-UCA concentration. The FLG mutations tested in this project include R501X and 2282del4, both of which stop protein translation within the first filaggrin repeat. Homozygous for these two mutations leads to pronounced AD phenotype (Weidinger et al., 2006). I have shown in Chapter 3 that NO directly induces functional Foxp3+Tregs with enhanced skin migration ability and in Chapter 4 that *cis*-UCA promotes Foxp3+Tregs differentiation indirectly by modulating human mo-DCs while decreasing CD4+ T cell proliferation and the percentage of potentially skin-homing CLA+ cells, therefore likely downregulates systemic inflammation.

Chapter hypothesis:

Based on these results, I hypothesize that besides vitamin D, NO and *cis*-UCA may contribute to the clinical efficacy of NB-UVB phototherapy for AD patients through their effects on T cells.

I tested this hypothesis by asking the following questions:-

- Does serum concentration of vitamin D or nitrate or FLG genotype correlate with clinical efficacy of NB-UVB phototherapy?

- Does UVB-therapy affect T cell phenotype and proportion of Tregs?

5.2 Results

5.2.1 Patients recruitment

The study was approved by the National Research Ethics Service (NRES) Committee North West – Preston (14/NW/1409). Patients were recruited from Department of Dermatology at University of Edinburgh. They were provided with a patient information sheet which explains the study in layman's terms and were given at least 24 hours to consider whether to participate the study. Then they signed a consent form before the start of study.

For inclusion in the study, patients had to have a clinical diagnosis of AD, be between the ages of 16 and 80 years and on the waiting list for UVB phototherapy.

The exclusion criteria were:

1. Treatment with systemic corticosteroids or other immune response modifying systemic drugs such azathioprine or methotrexate in four weeks before the study starts.
2. Taking aspirin or other NSAIDs in the week before each blood sampling.
3. Inability to give informed consent.
4. Pregnancy
5. Skin type 1 (always burns, never tans)
6. involved in current research or have recently been involved in any research prior to recruitment

In total, 11 male AD patients and 8 female AD patients were recruited, aged between 23~56. The severity of the AD was measured by SCORAD (SCORing Atopic Dermatitis) at the start and after eight weeks treatment. This is a composite measure of the area and intensity of AD objectively measured by the clinician, and a subjective measure of symptoms measured by

the patient. Blood samples were taken before and after an 8 week treatment. Plasma concentration of 25(OH) vitamin D and nitrate which is one of the end products of NO were analysed for all the recruited patients. FLG genotype was analysed by the Wellcome Trust Clinical Research Facility. The detailed method was described in 2.3.9. Whole blood staining for immune cell subset was performed for blood samples taken from these patients. Table 5.1 showed the patients information. Within the 19 patients recruited, 3 of them were FLG –null mutation carriers.

Table 5.1 Patients information.

Sample ID	Age	Gender	Score Before Treatment	Score at 8th Week	Improvement (Δ SCORAD)	Total UVB-dose (J/cm2)	FLG status
UVAD10	36	M	44.3	14.1	30.2	9.4	R501X het
UVAD11	39	M	46.1	14.5	31.6	8.8	WT
UVAD12	48	M	50.5	16.1	34.4	10.4	WT
UVAD13	40	M	27.5	15.4	12.1	15.9	WT
UVAD14	43	M	48.7	16.1	32.6	6.7	WT
UVAD15	30	M	26	9.9	16.1	8.8	WT
UVAD16	51	F	28.6	19.5	9.1	7.7	WT
UVAD17	32	F	32.6	13.6	19	14.5	WT
UVAD18	36	F	28.3	5.7	22.6	19.6	WT
UVAD19	56	F	52	14.5	37.5	15	2282del4 het
UVAD20	54	M	38.3	24.8	13.5	8.5	WT
UVAD21	50	M	73	27	46	11.5	WT
UVAD22	47	F	60.8	24.7	36.1	5.1	WT
UVAD23	23	F	55.9	17.5	38.4	8.9	WT
UVAD24	48	M	57.9	18.9	39	7.6	WT
UVAD25	26	F	54.1	17.3	36.8	15.5	R501X het
UVAD26	56	M	40.2	16.1	24.1	5.2	WT
UVAD27	48	M	55.7	12.3	43.4	7.2	WT
UVAD28	43	F	51.5	14.9	36.6	11	WT

As shown in Fig 5.1a, SCORAD, which was used in this study as an indication for AD severity, decreased in all patients after 8 weeks NB-UVB phototherapy. Under the ethics for this study, NB-UVB phototherapy was only given to patients who had phototherapy before and responded to the treatment or to those whose disease was self-reported to get better on exposure to sunlight. Therefore, this selection bias likely led to all patients recruited in this

study getting better after 8 week treatment. However, there was still some variation in the patients' responses to phototherapy. As shown in Fig 5.1b, although those with *FLG*-null mutations showed good improvement, there was no significant difference between AD patients with WT *FLG* compared to those with *FLG*-null mutations, which suggested that FLG genotype could not be used as a biomarker to predict the clinical efficacy of NB-UVB phototherapy. Besides FLG mutations, copy number variation with filaggrin also contributes to the risk of AD (Elias and Schmuth., 2009). Considerting the small number of AD patients with FLG mutations recruited, this would need to be repeated on a larger number to make any definite conclusion.

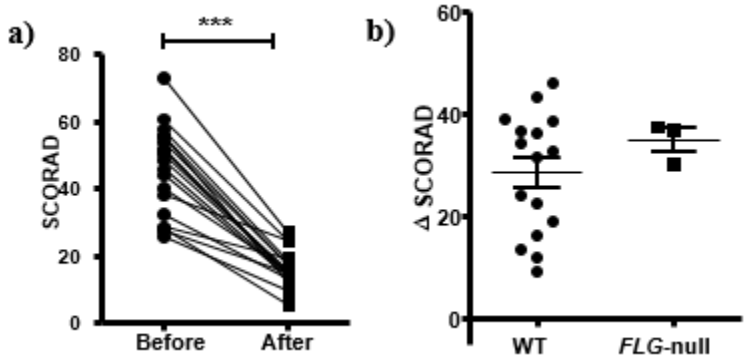


Fig 5.1 FLG genotype could not be used as a biomarker to predict the clinical efficacy of NB-UVB phototherapy. a) Severity of AD before and after 8 weeks NB-UVB phototherapy. b) Disease improvement in patients with wild type FLG and in patients with FLG null mutations. N=19 P value was calculated using Wilcoxon matched pairs T test.

5.2.2 Plasma 25(OH) vitamin D concentration was increased after 8week of NB-UVB phototherapy and changes of plasma concentration of 25 (OH) vitamin D did not correlate with disease improvement after NB-UVB phototherapy.

Plasma concentration of 25(OH) vitamin D was analysed by ELISA. As shown in Fig 5.2a, the concentration of 25(OH) vitamin D in plasma was significantly increased after NB-UVB

phototherapy. The correlation between changes of plasma concentration of 25(OH) vitamin D and disease improvement after NB-UVB phototherapy was analysed. As shown in Fig 5.2b, changes of plasma concentration of 25(OH) vitamin D did not correlate with disease improvement. These results suggested that vitamin D was not responsible for all the beneficial effect induced by phototherapy.

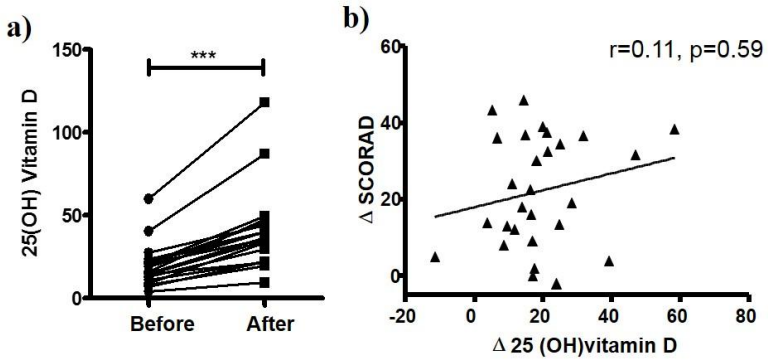


Fig 5.2 Plasma 25(OH) vitamin D levels was increased after NB-UVB phototherapy and changes of plasma concentration of 25(OH) vitamin D did not correlate with disease improvement. a) Plasma 25(OH) vitamin D concentration before and after treatment. b) Correlation between changes of 25(OH) vitamin D concentration and disease improvement. P values were calculated using the non-parametric Wilcoxon matched pairs test (Fig 5.2a) and Spearman rank correlation (Fig 5.2b). N=19.

5.2.3 Plasma nitrate concentration was not affected after 8 week of NB-UVB phototherapy and changes of plasma concentration of nitrate did not correlate with disease improvement after NB-UVB phototherapy.

Plasma nitrate concentration was analysed using Nitric Oxide (total) Assay Kit from Thermo Fisher (Fountain Drive, Renfrew, UK). As shown in Fig 5.3b, changes of plasma concentration of nitrate did not show any significant difference between before and after treatment and change in levels did not correlate with disease improvement.

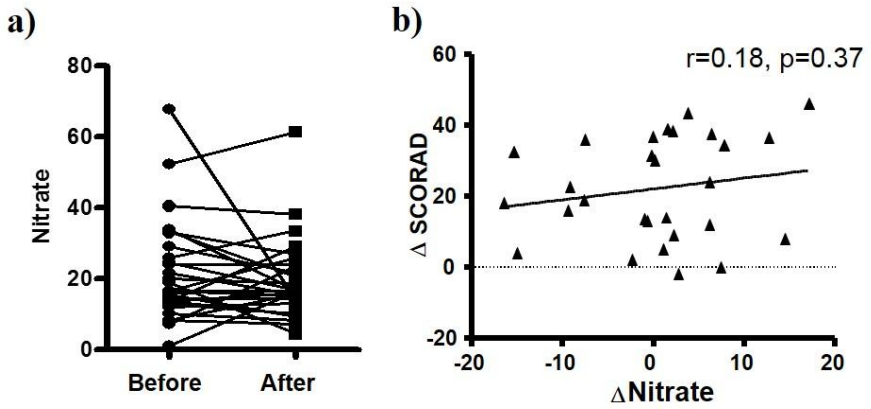


Fig 5.3 Plasma nitrate concentration was not affected after 8 week of NB-UVB phototherapy and changes of plasma concentration of nitrate did not correlate with disease improvement. a) Nitrate concentration before and after treatment. b) Correlation between changes of nitrate concentration and disease improvement. P values were calculated using the non-parametric Wilcoxon matched pairs test (Fig 5.3a) and Spearman rank correlation (Fig 5.3b). N=19.

5.2.4 Circulating CD4+CD25+CD127low/- Tregs and CLA+Tregs were not affected after NB-UVB phototherapy and the change of these two cell populations did not correlate with disease improvement.

To investigate whether UVB phototherapy affected the levels of circulating CD4+CD25+CD127low/- Tregs and especially of CLA+Tregs, and whether the changes of these subsets correlated with clinical efficacy of NB-UVB phototherapy, the percentage of these two cell populations in the whole blood were analysed by flow cytometry. As shown in Fig 5.4a, although half of the patients show an increase in Tregs after NB-UVB, this was not significant as such an increase was not observed for other patients. The change of CD4+CD25+CD127low/- Tregs did not correlate with disease improvement after 8 weeks of NB-UVB phototherapy. The percentage of skin homing CLA+Tregs was not affected by the

treatment and the changes of CLA+Tregs did not correlate with disease improvement either.

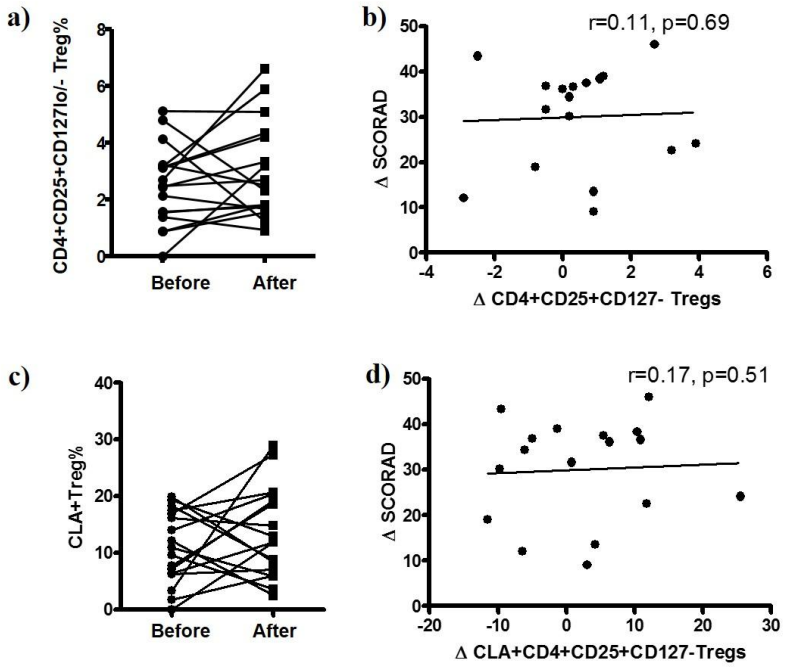


Fig 5.4 Circulating CD4+CD25+CD127low/- Tregs and CLA+Tregs were not affected after NB-UVB phototherapy and the change of these two cell populations did not correlate with disease improvement. After removing plasma, blood was diluted twice with ice cold PBS and stained with viability dye ef780. After viability staining, percentage of CD4+CD25+CD127low/- Tregs and CLA+Tregs were analysed using flow cytometry. a) Percentage of CD4+CD25+CD127low/- Tregs in whole blood. b) Correlation between changes of CD4+CD25+CD127low/- Tregs and disease improvement after NB-UVB phototherapy. c) Percentage of CLA+ CD4+CD25+CD127low/- Tregs in whole blood. d) Correlation between changes of CLA+CD4+CD25+CD127low/- Tregs and disease improvement after NB-UVB phototherapy. P value was calculated by Spearman rank correlation (N=19).

Besides regulatory T cells, other markers related with immune responses were analysed on total CD4+ T cells. Two of these markers were affected by NB-UVB phototherapy. As shown in Fig 5.5a, expression of the activation marker CD69 on CD4+ T cells was decreased after treatment. Expression of the skin homing marker CLA was also decreased after phototherapy

(Fig 5.5b). Taken together, these results indicated that markers related with CD4+ T cell activation and migration to the skin were decreased by NB-UVB phototherapy and the importance of these changes in determining clinical efficacy of NB-UVB phototherapy required further investigation.

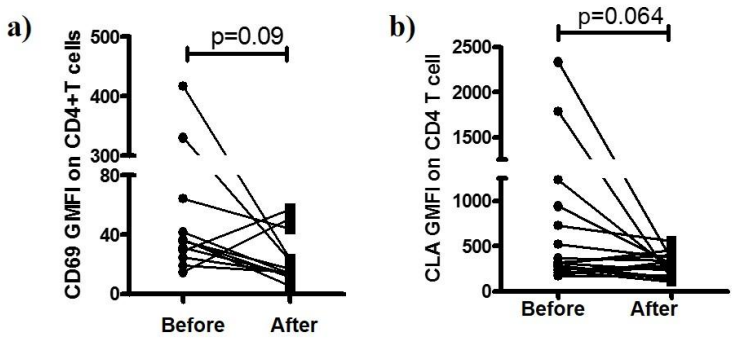


Fig 5.5 Markers reflecting CD4+ T cell activation and migration were decreased after NB-UVB phototherapy. After removing plasma, blood was diluted twice with ice cold PBS and stained with viability dye ef780. After viability staining, markers related with immune responses were stained and analysed using flow cytometry. a) Geometric mean of CD69 expression on CD4+ T cells in whole blood. b) Geometric mean of CLA expression on CD4+ T cells in whole blood. P value was calculated by a non parametric Wilcoxon paired test (N=19)

5.3 Discussion

Previous studies have shown that patients with AD respond differently to phototherapy. It has been shown that among 15 patients with AD treated with NB-UVB phototherapy, 66% improved and duration of therapy varied between 3 to 30 months. In Edinburgh, patients with AD were asked whether they tended to get better on exposure to sunlight, or with previous phototherapy before being selected for treatment with phototherapy. This selection bias may account for the fact that all patients recruited in this study improved after NB-UVB phototherapy. The second weakness of the current study was the UV dose that AD patients received was adjusted based on their biological responses during phototherapy since this was an observational study. Therefore, although patients had been through 8 week treatment, the total UV dose they received was different, which may contribute to the different immune responses in AD patients.

Here, I have shown that FLG genotype cannot be used as biomarker to predict clinical efficacy of NB-UVB phototherapy and changes in concentration of plasma 25 (OH) vitamin D and nitrate did not correlate with disease improvement. The number of recruited patients with *FLG* mutations in this study was low. Of 19 patients, only 3 carried *FLG* mutations. These 3 patients showed varied responses to phototherapy which suggested that *FLG* mutations did not affect patients' responses to NB-UVB phototherapy. An extension of the study with larger numbers of participants would be needed to confirm these results. Studies have shown that filaggrin expression negatively correlates with mast cell tryptase number in the nonlesional granular layer of AD patients (Ilves et al., 2015). It is also suggested that filaggrin deficiency associates with a defective antimicrobial barrier and increased colonization of Staphylococcus aureus which will further inhibits terminal differentiation of normal human keratinocytes by stimulating secretion of IL-6 (Elias and Schmuth, 2009).

Plasma vitamin D concentration was significantly increased after NB-UVB phototherapy although the changes in concentration of this molecule did not correlate with disease improvement which was consist with previous studies and further indicated that vitamin D was not responsible for all the clinical beneficial effect of NB-UVB phototherapy (Hart et al., 2011). Plasma concentration of nitrate was not affected after NB-UVB phototherapy. The change of nitrate concentration did not correlate with disease improvement either. One of the confounding factors for plasma nitrate concentration was diet. Nitrate is both a storage form for nitric oxide and an end product of its oxidation (Mowbray et al., 2009). The major source of nitrate in the circulation is from diet which it is not possible to control for in this pragmatic observational study. Overall levels of nitrate in the circulation might thus be an indicator for expected NO levels following UV radiation, but it is difficult to quantify overall NO activity from serum nitrate levels alone. Another possibility is NO reacts with environment and exerts its action locally. In humans, the concentration of nitrite and RSNO are 25 and 360 times higher in skin than in plasma. Adnana N. Paunel et al showed that upon exposure to UVA, nitrite and RSNO were photolysed to generate NO, the concentration of which was comparable or even higher than that produced by cytokine activated human keratinocyte cultured with maximal iNOS activity *in vitro*. NO rapidly diffuses and penetrates cell membranes with a diffusion distance of 500 μm in tissue (Paunel et al., 2005). In the context of the *in vitro* results described in Chapter 3 showing that NO directly promoted generation of Foxp3+ Tregs, local generation of NO may promote Foxp3+Tregs within the skin. To test this, further studies could be conducted by analysing the correlation between cutaneous concentration of nitrate and presence of Foxp3+Tregs in the skin. To detect the cutaneous concentration of NO, samples such as fluid from suction blister should be used. The third possibility is that NO generated by UV reacts with thiols containing protein in skin and the

Nitric Oxide Assay Kit which contains Griess Reagent can only detect nitrite and nitrate which leads to the underestimated concentration of UV-induced NO.

Tregs are important in the resolution of allergic immunity. As summarized in Chapter 1.3.5, reports about the number and function of Tregs in patients with AD remain controversial. In this study, the percentage of CD4+CD25+CD127low/- Tregs and CLA+ Tregs in whole blood were not significantly altered after treatment. This is consistent with previous studies showing that Foxp3+ Tregs in patients with AD were not modulated by NB-UVB phototherapy (Majoie et al., 2009). I have shown that the three UV irradiation induced molecules vitamin D, nitric oxide and *cis*-UCA all led to the generation of Foxp3+Tregs from CD4+ T cells either directly or indirectly as described in Chapter 3 and 4, however, the change in the proportion of CD4+CD25+CD127low/- Tregs in blood did not correlate with the change of SCORAD after treatment indicating that *in vivo* modulation of Tregs was complicated and interfering with anti-inflammatory molecules alone may not be enough to promote the generation of Tregs systemically. Tregs migration might contribute to this result as well since I have shown that CD4+ T cells migration was affected by UV-induced NO and *cis*-UCA leading to no drastic changes of Tregs was observed in the blood. These results also confirm that it is important to investigate the presence of Foxp3+Tregs in the skin before and after NB-UVB phototherapy to see whether it is correlated with patients' response to phototherapy. The small number of Tregs circulating in blood meant that the function of Tregs before versus after treatment could not be tested in this study which may be important for evaluation of the clinical efficacy of NB-UVB phototherapy.

Tissue-selective trafficking of T lymphocytes is mediated by a complex interplay between cytokines and chemokines with adhesion receptors. It has been shown in psoriasis that the

number of T cells infiltrating the skin is correlated with disease activity (Schön et al., 2003). Therefore, prevention of T cells migration to skin appears to be an attractive target to treat inflammatory skin diseases. Here, I have shown that the expression of CLA on CD4+ T cells was decreased after treatment, which suggested that skin migration of CD4+ T cells was modulated by NB-UVB phototherapy. However, a previous study has shown that blocking E-selectin using specific antibody did not improve psoriasis in a clinical trial (Bhushan, Bleiker et al. 2002). This suggests that although CLA is important in T cell migration, interfering with a single selectin alone may not be enough to prevent the inflammatory responses in AD. Therefore, further studies investigating chemokines and other adhesion molecules such as integrins that are related with CD4+ T cell skin migration are required.

Summary

- All donors improved after NB-UVB therapy

- Concentration of circulating 25 (OH) vitamin D, but not of nitrate, was increased after NB-UVB therapy.

- FLG genotype cannot be used as biomarkers to predict AD patients' responses to NB-UVB phototherapy.

- Changes in the concentration of 25(OH) vitamin D and nitrate did not correlate with disease improvement after NB-UVB phototherapy.

- Markers related with CD4+ T cell activation and skin migration were decreased after NB-UVB phototherapy.

Chapter 6 General Discussion

6.1 Factors determining AD patient response to UV-B therapy.

Although it is commonly referred to as a single disease, recent studies suggest that AD can be categorized into different subtypes based on disease onset, immune polarization and gene variant (Leung and Guttman-Yassky 2014). Studies have shown that different clinical phenotypes of AD are associated with distinct activation or suppression pattern of immune responses. For example, there are distinct differences in cytokine production in patients with intrinsic versus extrinsic AD, which indicates that these patients may respond differently to the same treatment (Suárez-Farinas, Dhingra et al. 2013).

Within the last two decades, NB-UVB therapy with wavelengths of 311 nm has turned out to be a major therapeutic strategy for a variety of skin diseases. In the clinical study conducted in my thesis, all patients improved after 8 weeks of treatment due to the pre-selection of patients undergoing NB-UVB phototherapy in the Dermatology department in Edinburgh. Still the patients exhibited different extent of responses for NB-UVB phototherapy in AD which is consistent with previous studies (Mok, Koh et al. 2014).

Although NB-UVB phototherapy has been recognized for its good safety profile regarding acute adverse events, concerns remain regarding long term use. Risk of developing skin cancer after NB-UVB phototherapy is not the main issue, as evidenced by Osmancevic *et al* who investigated the risk of skin cancer in 160 psoriasis patients who had been treated with UVB for more than 100 times during the past 5 years and showed that the risk for developing skin cancer in UVB-treated patients is similar to the general population (Osmancevic, Gillstedt et al. 2014). Similar results have been published for children aged 16 or under (Sen, Rifaioglu et al. 2014). AD patients who are prescribed phototherapy need to visit the specific

phototherapy department every time and doctors with special training are required. Some patients cannot endure the associated heat and sweating and others have photosensitivity or co-existing polymorphous light eruption (Walling HW et al., 2010). Therefore, it is would be helpful to be able to identify those patients who will not respond to the treatment to avoid these issues for patients who are less responsive to NB-UVB phototherapy.

Therefore, I set out to determine the factors that contribute to the clinical efficacy of NB-UVB phototherapy.

6.1.1 FLG Genotype

Gene variants are reported to affect clinical efficacy of NB-UVB phototherapy. It is suggested by Dai *et al* that single-nucleotide polymorphisms in genes related with nucleotide excision repair pathways that contribute to repair DNA photoproducts are associated with the efficacy of NB-UVB therapy in Chinese patients with active vitiligo (Dai, Zhou et al. 2015).

Within the study performed during my thesis, 19 AD patients were recruited and only 3 of them were FLG null mutation carriers. These 3 patients showed medium responsiveness to the treatment which indicates that FLG mutations do not affect clinical efficacy of NB-UVB phototherapy. One confounding factor for this study was the UV dose AD patients received. Although they were all treated with NB-UVB phototherapy for 8 weeks, the total dose of UV irradiation they received was different, as adjusted according to their biological responses. Further studies comparing AD patients with and without FLG null mutations receiving same dose of NB-UVB irradiation are necessary to determine the effect of FLG mutations on the clinical efficacy of NB-UVB phototherapy.

Although FLG null mutations in this study did not correlate with clinical efficacy of NB-UVB phototherapy, FLG status may still provide useful information in determining the best treatment for AD patients. FLG null mutations result in barrier defects and enhanced allergen penetration as summarized in the Introduction 1.1.3.2. Decreased suppressive function of Tregs and increased resistance of effector T cells to the modulation of Tregs both contribute to the loss of immune suppression seen in the presence of strong stimulation (Baecher-Allan et al. 2001). Studies have shown that repetitive stimulation of Tregs through TCR/CD28 rendered them less suppressive and more inflammatory by secreting IL-17 (Beriou et al. 2009). More importantly, the migratory behaviour of these Tregs was also affected leading to accumulation of the non-functional Tregs in the skin (Rappl, Schmidt et al. 2008). Therefore, skin barrier improving therapy may contribute to the resolution of AD through several mechanisms including prevention of allergen penetration and enhancing Tregs function. Taken together, the combination of skin barrier improving therapy and NB-UVB phototherapy may be more effective.

6.1.2 Factors reflecting immune responses

Distinct immune-polarized pathways exist in various AD subsets, which may affect the clinical efficacy of treatment. For example, significantly enhanced expression of IL-17, IL-22 and IL-23 have been reported in intrinsic AD patients with normal IgE level, which suggested that these patients were potentially more responsive to suppression of the IL-17/IL-23/IL-22 polarized immune axes. Therefore, IgE concentration can be used to determine the effective treatment for AD (Leung and Guttman-Yassky 2014). This evidence suggests that development of biomarkers to assess the immune polarization in AD may offer valuable information in determining proper treatment in AD.

Here, I have shown that systemic 25 (OH) vitamin D concentration was significantly increased after NB-UVB phototherapy, as previously reported. However, the change in 25 (OH) vitamin D did not correlate with disease improvement, which confirmed my original hypothesis that vitamin D was not responsible for all the clinical beneficial effect of NB-UVB phototherapy. Systemic nitrate concentration was analysed as well. Nitrate concentration was not affected after treatment and the change of systemic nitrate did not correlate with disease improvement either. Taken together, these results suggested that systemic 25 (OH) vitamin D and nitrate could not be used as a biomarker to predict clinical efficacy of NB-UVB phototherapy.

6.2 NB-UVB phototherapy in AD: systemic effect vs local effect

The exact mechanism of NB-UVB therapeutic action remains poorly understood and it is not clear whether local or systemic effect is more important. It is suggested that NB-UVB phototherapy improves psoriasis through local effects (Dawe, Cameron et al. 2002). Ekman *et al* reported that although NB-UVB therapy improves skin symptoms, it did not affect chemokine level in serum from psoriasis vulgaris patients. Chemokine production of PBMC activated with either LPS or anti CD3/28 was not affected by NB-UVB therapy either, leading to the conclusion that the effect of NB-UVB may be more focused on local inflammation than on systemic (Ekman, Sigurdardottir et al. 2013).

Here I showed that the expression of markers reflecting T cell activation CD69 on CD4+ T cells in blood was decreased ($p=0.09$) after NB-UVB phototherapy in AD, which suggested a systemic inhibition of CD4+ T cell activation after treatment. The expression of CLA, a marker related to CD4+ T cell migration, was also down-regulated ($p=0.064$) after NB-UVB phototherapy, which may decrease CD4+ T cell migration into skin. AD as a chronic

inflammatory skin disease is characterized by the accumulation of lymphocytes in skin. The recruitment of lymphocytes is suggested to be mediated by the combined actions of chemokines and interaction between lymphocytes and endothelial cells mediated by cell adhesion molecules (Leung, Boguniewicz et al. 2004). Therefore, the decreased systemic CD4+ T cell activation and skin migration may contribute to the clinical beneficial effect of NB-UVB phototherapy. This is consistent with a previous study reported by Yule *et al* that NB-UVB phototherapy improved AD through both local and systemic effects and a variation in which response dominated existed between patients (Yule, Dawe et al. 2005). Taken together, these results indicate that NB-UVB phototherapy improves AD at least partially through systemic effect.

6.3 Regulatory T cells

It is well known that UV irradiation induces the production of vitamin D, which is suggested to be responsible for the immunosuppressive effect of UV irradiation. However, UV irradiation has been shown to suppress murine model of multiple sclerosis independently of vitamin D production (Becklund, Severson et al. 2010). Other studies showed that changes in vitamin D production in skin by UV irradiation have not been correlated with local immune regulation (Hart, Gorman et al. 2011). Taken together, these results suggest that vitamin D has similar immunosuppressive function as UV irradiation but does not account for all the effects of UV induced immunosuppression.

Therefore, I studied the *in vitro* effect of two UV-induced anti-inflammatory molecules NO and *cis*-UCA on human peripheral CD4+ T cells and mo-DCs phenotype and function. Regulatory T cells are important in the resolution of allergic immunity as summarised in

Chapter 1.2. So I focused on the effect of these two molecules on Tregs differentiation and function.

As shown in Chapter 3, NO did not affect the phenotype of human mo-DCs and exerted its effect on human peripheral CD4+ T cells directly. NO increased the proportion of CD25+Foxp3+CD127- T cells from total CD4+ T with enhanced expression of CLA indicating their ability to migrate to the skin. The MFI of Foxp3 on the induced cell population was also increased by NO which indicated their enhanced suppressive effect. The effects of NO on CD4+ T cells were mediated by cGMP. The increased proportion of CD25+Foxp3+CD127- T cells was not due to lesser proliferation of other T cell populations such as the Foxp3- T cells since NO inhibited the proliferation of both cell populations to a similar extent. I did not test the final number of Foxp3+ cells after culture, therefore, one possibility I cannot rule out was NO selectively killed Foxp3- T cell population leading to the increased proportion of Foxp3+ cells. But the results that NO also enhanced the proportion of CD25+Foxp3+CD127- T cells from CD4+CD25- cells further confirmed that NO could induce the generation of CD25+Foxp3+CD127- Treg cells. Besides, the ratio of effector T cells and Tregs is more important than the actual number of Foxp3+Tregs in determining the outcome of an immune response in humans, therefore, the increased proportion of CD25+Foxp3+CD127-cell may be of more clinical relevance.

In contrast to mouse CD4+ T cells, TGF-β or activation induced Foxp3 expression in human CD4+ T cells does not confer suppressive function of Tregs (Tran, Ramsey et al. 2007). Therefore, the suppressive function of NO-induced CD25+Foxp3+CD127-cells was further analysed. Results showed that NO-induced Foxp3+ Tregs were functional as evidenced by inhibiting the proliferation of autologous CD4+ T cells. The suppressive function of NO-

induced Foxp3+Tregs were mediated by membrane bound TGF-β and PD-1/PD-L1 but not CTLA-4. The cytokines IL-10 secreted by NO-treated CD4+ T cells was not sufficient for the suppressive function of NO-induced Fopx3+Tregs.

The phenotype of UV-induced Tregs has been summarized in Chapter 1.4.1.3. UV-induced Tregs express functional CTLA-4 and secrete high levels of IL-10 and TGF-β. It is suggested that the immunosuppressive function of UV-induced Tregs is partially mediated by CTLA-4. In a murine contact hypersensitivity model, transferring UV-induced Tregs renders recipient mice unresponsive, the effect of which is lost by injecting anti CTLA-4 Ab (Schwarz, Beissert et al. 2000). Taken together, this suggests that NO-induced Fopx3+Tregs are not equivalent to UV-induced Tregs and UV irradiation might induce the generation of Foxp3+Tregs through multiple mechanisms. Alternatively, this could also be due to intrinsic differences between mice and humans.

In order to investigate the correlation between nitrate and Tregs *in vivo*, the percentage of Tregs in AD patients' blood before and after NB-UVB phototherapy was investigated. However, the percentage of Tregs was not consistently increased in AD patients after 8 week treatment. There might be two possible reasons for this. Firstly, previous studies have shown that local proliferation of Tregs can happen within the skin. It is reported by Clark that natural Tregs and their precursors existed in human skin and could be expanded by dermal fibroblasts in combination with IL-15 (Clark and Kupper 2007). As summarized in Chapter 3.1, the concentration of nitrogen species, which will release NO upon exposure to UV irradiation, is much higher in skin than in plasma. Therefore, it is possible that systemic concentration for NO is not high enough to generate Foxp3+Tregs and NO induced Foxp3+Tregs happen locally in skin. Secondly, it has been suggested that keratinocytes produce chemokines that induce the migration of Tregs.

Considering that NO-induced Foxp3+ Tregs have higher expression of CLA, it is possible that these Foxp3+Tregs already migrated to skin and cannot be detected in blood. The importance of Foxp3+Tregs in AD have been reported in previous studies showing that despite the presence of IL-10 secreting Tr1, the dys−regulated disease causing effector T cells was still observed in AD lesions with impaired CD4+CD25+FoxP3+ T-cell infiltration (Verhagen, Akdis et al. 2006).

In Chapter 4, the effect of *cis*-UCA on human peripheral CD4+ T cells was analysed. *Cis*-UCA exerted both anti-inflammatory and pro-inflammatory effect on CD4+ T cells. *Cis*-UCA may contribute to suppression of skin inflammation by decreasing the proportion of CLA+ T cells of total CD4+ T cells that have been activated with plate-bound anti-CD3/28 for 5 days. *Cis*-UCA also inhibited inflammation by decreasing CD4+ T cell proliferation, phosphorylation of ERK signalling and increasing secretion of anti-inflammatory cytokines IL-10 and CCL4 which will contribute to the recruitment of Tregs. On the other hand, *cis*-UCA may promote inflammation by decreasing the proportion of CD25hi Fopx3+ cells and affecting vitamin D's effect via decreasing VDR expression, which suggested that there might be a fine balance between UV-induced anti-inflammatory molecules.

Besides its direct effect on peripheral CD4+ T cells, *cis*-UCA may affect CD4+ T cell phenotype and function indirectly through antigen presenting cells. Therefore, the effect of *cis*-UCA on human mo-DCs was also investigated. Results showed that *cis*-UCA decreased the expression of antigen presenting molecules HLA-DR and co-stimulatory molecules CD86 and CD40 on immature mo-DCs. The immune stimulatory effect of *cis*-UCA treated immature mo-DCs was analysed by co-culturing with allogenic CD4+ T cells. Results showed that *cis*-UCA treated immature mo-DCs were indeed less immune stimulatory as evidenced by promoting generation of CD25+Foxp3+CD127- cells when co-cultured with

allogenic CD4+ T cells. Study has shown that the threshold of antigen to active Foxp3+ Tregs is estimated to be 10-100 times lower than that is needed to activate naïve T cells (Sakaguchi, Yamaguchi et al. 2008). This implies that DCs with low expression levels of CD80/CD86 preferably activate Tregs than naïve T cells. However, the expression of antigen presenting molecules and co-stimulatory molecules on LPS-matured mo-DCs were not affected by *cis*-UCA and *cis*-UCA treated LPS-mature mo-DCs did not affect the proliferation and the proportion of CD25+Foxp3+CD127- cells when co-cultured with allogenic CD4+ T cells. Considering LCs as the major antigen presenting cells in the epidermis where the concentration for *cis*-UCA is relatively high are mainly immature antigen presenting cells, these results suggest that *cis*-UCA's effect on antigen presenting cells is more important in maintaining the homeostasis of skin by keeping immature DCs even less immune stimulatory while it may not be strong enough to suppress the function of matured antigen presenting cells.

6.4 Conclusion

Vitamin D is not the only anti-inflammatory molecule induced by UV irradiation and is not responsible for all the beneficial effects of NB-UVB phototherapy in treating patients with AD. UV irradiation also photolyses nitrogen-species to release NO and converts trans-UCA to immune suppressive *cis*-UCA in the skin. This study described a novel function of NO to directly induce functional Foxp3+Tregs with enhanced skin migration ability which mediate their suppressive function through membrane bound TGF β and PD-1/PD-L1 but not CTLA-4. *Cis*-UCA has both anti- and pro-inflammatory effect on human peripheral CD4+ T cells. *Cis*-UCA suppressed inflammation through decreasing the percentage of CLA+ cells, inhibiting CD4+ T cell proliferation, inhibiting ERK phosphorylation and increasing IL-10 and CCL4 secretion while it might promote inflammation by decreasing percentage of CD25hi Foxp3+ Tregs and decreasing expression of VDR

which may affect the immune suppressive function of vitamin D. *cis*-UCA also affects human mo-DCs phenotype by decreasing the expression of HLA-DR and co-stimulatory molecules CD86 and CD40 rendering these cells less immune stimulatory as evidenced by enhanced generation of CD25+Foxp3+CD127- cells when co-cultured with allogenic CD4+ T cells.

The clinical study first investigated the mechanisms responsible for the clinical efficacy of this treatment, and I have shown that expression of CLA and CD69 on CD4+ T cells was decreased after 8 week NB-UVB phototherapy suggesting lower activation and migration to the skin of activated T cells. The study was also made to potentially identify biomarkers that could be used to predict patients' responsiveness to NB-UVB phototherapy. The results suggest that FLG genotype and plasma concentration of 25 (OH) vitamin D and nitrate could not be used as biomarkers to predict clinical efficacy of NB-UVB phototherapy.

Considering the sample size in this study, further studies with larger number of participants will be required to confirm the conclusion that FLG mutations do not affect clinical efficacy of NB-UVB phototherapy. Besides, in order to further determine the effect of FLG mutations on the clinical efficacy of NB-UVB phototherapy, AD patients with and without FLG null mutations receiving same dose of NB-UVB irradiation are needed. Lastly, further study investigating the correlation between the presence of Foxp3+Tregs locally in the skin before vs after NB-UVB phototherapy and cutaneous concentration of nitrate/nitrite will provide more information of the mechanisms contributing to the clinical efficacy of NB-UVB phototherapy.

Overall, the study has elucidated the key cellular effects of UV irradiation induced anti-inflammatory molecules NO and *cis*-UCA on the generation of Tregs which will be of importance when considering future NB-UVB therapies for patients with AD or other autoimmune diseases that can

benefit from phototherapy.

Appendix I Patients information sheet for the clinical study

Department of Dermatology
Lauriston Building
Lauriston Place
Edinburgh EH3 9HA

The effect of ultraviolet on Interleukin 22 production by immune cells in atopic dermatitis

Introduction

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what will be involved. Please take time to read the following information sheet carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you would like to take part in the study. All the information collected about you during the course of the research will be kept strictly confidential. Your General Practitioner will be informed of your participation in this study, provided that you are in agreement with this.

What is the purpose of the study?

Atopic dermatitis (AD)/Eczema is one of the most common skin conditions and affects more than 1 in 5 people in the UK. It is a condition causing a red, itchy rash. The exact cause of eczema is poorly understood, and we are carrying out research on this. In about 80% of eczema patients, natural sunlight or artificial sunlight makes their skin improve. How well and how quickly patients respond can vary and we wish to study why this is. Interleukin 22 is a substance that is produced by immune cells. Patients with high levels of this substance in their blood typically have worse eczema than those with low levels. It is thought that improvements in eczema patients following sunlight exposure may be due to sunlight

reducing levels of Interleukin-22. We wish to study how treatment with artificial sunlight (phototherapy) affects levels of Interleukin-22 in eczema patients.

Why have I been invited?

You are on the waiting list for ultraviolet (phototherapy) or systemic treatment of your skin.

Do I have to take part?

No. It is entirely up to you to decide. You have been sent a copy of this information sheet to read through, think and ask any questions before making a decision. If you do decide to take part you will be asked to sign a consent form and given a signed copy of the consent form to keep. If you decide to take part you are still free to withdraw at any time without giving a reason. A decision to withdraw or not to take part, will not affect the standard of care you receive.

Will my taking part in the study be kept confidential?

Yes, it will be kept confidential. All the information collected about you during the course of the research will be kept strictly confidential. Your General Practitioner will be informed of your participation in this study, provided that you are in agreement with this.

What will happen to me if I take part?

At your first treatment visit, the doctor running this study will ask if you would like to participate and answer any questions you might have concerning the study. You will be given a minimum of 24hours to consider it. If you are happy to be included in the study he or she will ask you to sign a consent form. You will attend for treatment as normal (e.g. twice per week for phototherapy) and will receive study-specific measures at the beginning of treatment and following 8 weeks (i.e. there will be 2 research visits). The doctor will then examine your skin to assess the severity of your eczema. The doctor will also take a 30 ml (about 6 teaspoons) blood sample. We would also like to take 4 small samples (biopsies) of your skin at each visit. At each visit, 2 skin biopsies samples will be taken from skin affected by eczema and 2 samples will be taken from skin not affected by eczema. Four biopsies will be taken at each visit (with a total of 8 skin biopsies taken over the course of the studyAt each visit we would also like to take 10 small skin samples using a tape strips. A again these will be taken from normal and affected skin. The doctor will ask you about your eczema and how you respond to sunlight. Each of the 2 visits will last around 1 hour.

What are the possible disadvantages and risks of taking part?

There are no particular risks attached to this study. Taking blood is a routine clinical

procedure. Occasionally people are left with a temporary bruise after blood is taken. The clinical scoring of your eczema will take about 10 minutes. Skin biopsies will be performed under local anaesthetic, which will require a small injection near the site of the biopsy. The injection of local anaesthetic before the biopsy often may sting. The biopsy sites will be closed with a suture ("stitch") to each site. The sites will heal over two to four weeks, and leave a small (3 to 4 mm) scar. there may be some discomfort while the biopsy heals. Sutures will be removed by Dr Weller or Dr Pappa. This will not require an additional visit to the clinic. Suture will be removed as part of a routine visit. This almost invariably heals well, but there is a theoretical risk of keloid scarring, or poor scarring due to infection. Keloid scarring is extremely unusual in the forearm, where these studies will be carried out. The biopsies will be undertaken using aseptic technique, so that the risk of wound infection is very low.

Tape stripping involves pressing a strip of sellotape like tape onto the skin and pulling it off, to remove some surface skin cells Tape stripping is painless and leaves no marks. There will be no alteration in the phototherapy treatment of your eczema.

What are the possible benefits of taking part?

None personally, but the research we are undertaking should help the understanding of eczema and we hope will lead to better treatments for all in future.

What will happen to my blood and skin tissues?

We wish to analyse your blood and skin samples to measure levels substances associated with eczema to see how these change following treatment. Some of these tests will involve DNA analysis of your blood.

This will be fully anonymised, so that DNA analysis results cannot connected to you.

We would like to keep any remaining samples to use in future studies.

If you lose the capacity to give consent, you would be withdrawn from the study. In these circumstances, we would like to keep any data or tissue we have already collected. However, no further data or tissue would be collected.

Who will see my records and know about my taking part?

If you consent to take part in the research your medical records may be inspected by the regulatory authorities to check that the study is being carried out correctly. Your name will not be disclosed outside the clinic or appear on any reports or publications resulting from the study. With your permission your GP (family doctor) will also be informed that you have

taken part in this research study.

The data generated from this research will be stored on a password protected computer and will be anonymous, with no indication of the identity of individuals involved. The data will be kept for 5 years or until published in the scientific literature (whichever is the sooner).

What will happen to the results of the research study?
The study may be published in a scientific journal, usually within three years after completion of the study. If results are published, this will be done without revealing your identity, which will remain confidential.

Who is organising the study?
The research study is being conducted by doctors at the University of Edinburgh Dermatology department. Your doctor will receive no personal payment.

Who has reviewed this study?
The study was approved by the NRES Committee North West - Preston.

If you need any more information about this study please contact:

Dr Richard Weller 0131-536-3229 or r.weller@ed.ac.uk

If you would like to discuss this study with someone independent of the study team please contact: Professor Jonathan Rees on: (0131 536 2041) or email: k.muir@ed.ac.uk

If you wish to make a complaint about the study please contact NHS Lothian:

NHS Lothian Complaints Team
2nd Floor
Waverley Gate
2-4 Waterloo Place
Edinburgh
EH1 3EG
Tel: 0131 465 5708

Thank you for taking time to read this information. If you decide to take part in the research study, you will be given a copy of this leaflet for your information.

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